AUG 4 1924

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XL, No. 2 AUGUST 1, 1924

PUBLISHED MONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

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WAVERLY PRESS
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BALTIMORE, U. S. A.

LEAD STUDIES.

III. The Effects of Lead on Red Blood Cells.

Part 1. Changes in Hemolysis.

By JOSEPH C. AUB, M.D., PAUL REZNIKOFF, M.D.,* and DOROTHEA E. SMITH.

(*From the Laboratories of Physiology of Harvard Medical School, Boston.*)

(Received for publication, March 27, 1924.)

Changes in the blood are often the first and most striking signs of lead poisoning in man. It is therefore probable that lead acts specifically upon the blood system. By studying the mechanism of this action two things might be learned: (1) the physiological, physical, and chemical reactions of lead on isolated cells, which would probably indicate its action on other body cells, and (2) the mechanism of the production of at least one type of secondary anemia. Studies of these problems are reported in this series of papers.

Much work on the subject of lead anemia has already been reported. This sign of the disease was probably first recognized by Laennec (1) who described its clinical aspects in 1831. Since then a vast amount of literature has dealt chiefly with the pathological picture, or the stippling of red blood corpuscles. Very few studies of the mechanism of the reaction have been published.

That lead causes marked anemia in man has been repeatedly demonstrated. Exposure of experimental animals is also followed by the same changes, as has been shown, in the case of rabbits, after intramuscular injection by Carcanaque and Maurel (2) and after oral administration by Key (3).

This anemia has been attributed either to marked destruction of blood or to a lesion in the bone marrow and a consequent deficient blood formation. Evidence for both processes appears in the literature. Stockman and Charteris, and Raimondi (4) observed in the bone marrow of rabbits a marked preliminary increase of leucoblasts with a disappearance of fat, which was later followed by definite gelatinous degeneration. Wolff (5) studied a case of severe lead poisoning in which the bone marrow indicated attempts to regenerate blood followed by deficient hematopoietic function. Meillère (6), Schnitter (7), and Sellers (8) thought lead definitely injured both blood corpuscles and bone marrow. But

* Fellow, National Research Council.

even though a late degeneration of the bone marrow may occur in lead poisoning, the primary appearance of anemia is probably due to abnormal *destruction* of the cells in the circulating blood—a fact first suggested by Bouchard (9), and later mentioned by various other writers including Rauch (10). The evidence for this is diverse. In early lead intoxication there is a hyperplasia of bone marrow (4); and many nucleated red cells in the circulation indicate regeneration. Key (3) has shown that within 24 hours after the ingestion of 1 gm. of lead the number of circulating red blood cells in rabbits decreased more than 20 per cent, and that many nucleated red cells appeared. During the onset of lead anemia Meillère (6) was able to show that the urinary excretion of iron, which is largely derived from red corpuscles, definitely increased. Working in this laboratory, Dr. J. Brady (11) has demonstrated that slow intravenous injection of lead acetate dissolved in saline solution causes marked hematuria and very marked increase in the excretion of bile pigments from the common duct. These unpublished experiments, which were performed on rabbits, indicate definitely and very clearly that small quantities of lead in the blood stream cause great destruction of blood, which could not be accounted for by any osmotic changes. In human cases Dr. Chester Jones (11) has obtained similar evidence. He found that the bile obtained by duodenal tube from several of our cases of plumbism, as well as the blood plasma, contained very high concentrations of bile pigments. All of these facts indicate the occurrence of marked peripheral destruction of blood caused by the ingestion of lead. But no good explanation for this increased destruction of blood has been previously suggested, because there has been very little thorough experimentation upon the subject.

Several phenomena associated with the blood in lead poisoning have, however, been noted. Since 1899, when Behrend (12) first observed the stippling associated with lead poisoning a great many reports have appeared in the literature. These have recently been summarized by Key (3). It seems probable that stippled cells are really young red corpuscles which are degenerating as a result of exposure to lead. But the mechanism of their development is not yet understood, and is particularly difficult to outline because stippling cannot be produced *in vitro* (3, 13).

Another change in red blood cells in lead poisoning is their increased resistance to hemolysis in dilute saline solution. As long ago as 1873 Malassez (14) reported not only that anemia was a symptom of lead poisoning, but that the blood cells were larger and more fixed than normal. Agasse-Lafont and Heim (15) are said to have found slightly increased globular resistance in cases of plumbism. von Liebermann (16) also demonstrated this well by a simple test which has been confirmed by Orbán (17) and Hayhurst (18). Fici (19) who has recently studied this reaction *in vitro*, found that lead acetate exerts a striking effect on the hemolysis of red cells in hypotonic salt solutions—which varies with concentration of lead and with temperature. His observations have been confirmed in some of the experiments reported here which were designed to investigate the action of lead on red blood cells *in vitro*. An explanation for this phenomenon has not, however, been advanced.

As the first step in a study of hemolysis by lead an investigation of the action of its salts on red blood cells *in vitro* was made.

Methods.

Unless otherwise stated, the blood was gently defibrinated and the red blood cells were freed from plasma by washing three times in carbonate- and phosphate-free Ringer solution.[1] Enough of this Ringer solution was then added to the cells to make the volume that of the original quantity of whole blood. This suspension was divided into fractions, one of which served as control. Lead or other metallic salts[2] in Ringer solution were added to the other fractions, allowed to stand for 1 hour, and then washed off with an excess of Ringer solution. The control was treated similarly but with Ringer solution alone. Comparison of this control with the other fraction showed the results of the experiment. In every case the pH of all solutions was carefully kept between 6.4 and 6.8, and nearly always at 6.5. This range of variation produced no demonstrable changes in our results.

All manipulations were so standardized that they did not vary from day to day. In every experiment the control tests allowed direct comparisons—a method which gives more accurate results than would comparisons with an average normal.

The so called "fragility," or hypotonic, tests of hemolysis were made in the usual manner. 0.1 cc. of washed blood cells was added to 1 cc. of hypotonic sodium chloride solution. The difference in the concentration of salt in adjacent tubes was 0.025 per cent. The results proved to be similar whether NaCl, Ringer, or blood serum dilutions were used. Therefore, simple sodium chloride solutions were employed because of ease in preparation; but it is certain that the absence of calcium or other ions plays no significant rôle in the results which were obtained after the tubes had stood for 15 hours in the cold room. The degree of hemolysis was determined from a set of standard tubes which contained known dilutions of the test blood and distilled water. The amount of hemolysis in a given saline concentration could thus be recorded as a percentage of the total. To check the readings in these tubes the amount of unhemolyzed cells was carefully observed. 100 per cent hemolysis is indicated by a clear solution without a sediment of intact red cells. This method is quite accurate in tubes where hemolysis is less than 60 per cent, but where it is greater than that an error of 10 per cent must be allowed.

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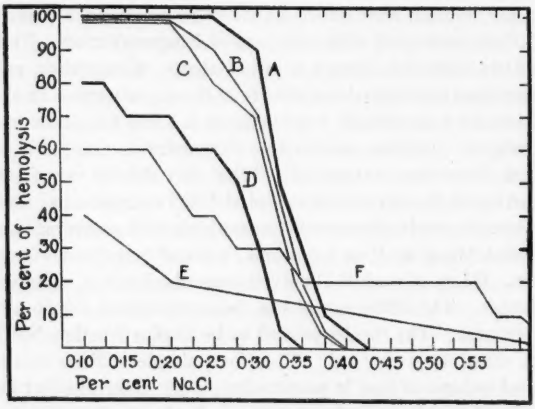
[1] The Ringer solution was made up as follows: NaCl, 0.9 per cent, KCl, 0.042 per cent, $CaCl_2$, 0.024 per cent. No carbonate or phosphate was added, to avoid the formation of their relatively insoluble lead salts. The pH was kept at 6.5 throughout in order to avoid the formation of lead hydroxide.

[2] The concentration of these salts is expressed as the weight of the metallic constituents and is so very low—approximately 1 part per 100,000—that no demonstrable changes in osmotic tension can be ascribed to these variations. Where higher concentrations are used, the concentration of NaCl is proportionally reduced.

OBSERVATIONS.

When normal red blood cells were exposed to varying concentrations of lead as lead chloride for 1 hour, the variations in their resistance to these hypotonic saline solutions were very marked (Text-fig. 1). Cells exposed to a concentration of only 2 parts of lead by weight per million showed a definite increase in resistance, while those in a solution of 1 part per 100,000 showed so marked an



TEXT-FIG. 1. Sodium chloride hemolysis. The effect of varying the quantity of lead. The curves show the effect on hemolysis of previous exposure for 1 hour of washed red blood cells to:

B, 1 cc. of red blood cells exposed to 0.002 mg. of Pb as chloride.
C, 1 " " " " " " " 0.004 " " " " "
D, 1 " " " " " " " 0.01 " " " " "
E, 1 " " " " " " " 0.03 " " " " "
F, 1 " " " " " " " 0.08 " " " " "

A shows the action of the control normal red blood cells in Ringer solution. This chart indicates the per cent of hemolysis in each concentration of salt solution.

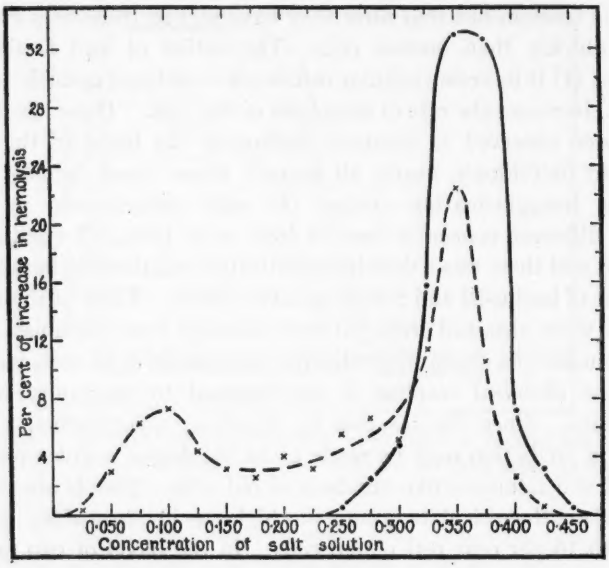
effect that many of the cells were not even completely hemolyzed in 0.1 per cent saline solution, and some remained unhemolyzed in 0.05 per cent saline. Normal cells, on the other hand, averaged complete hemolysis in 0.25 per cent salt solution. It is striking that in three tubes, corresponding to those in which marked destruction of normal cells first appeared, there was no hemolysis of the least resistant

"leaded" corpuscles. When greater concentrations of lead are used (Text-fig. 1) two phenomena become evident. The resistance to hemolysis increases; but some cells are so injured that they hemolyze upon standing even in normal Ringer solution, although the controls do not. These facts are still more sharply demonstrated in tubes of hypotonic saline where a dark ring of hemolysis lies directly over the cellular sediment, and the supernatant fluid is relatively clear. This indicates that there is no prompt hemolysis on exposure to diminished osmotic tension, but that after they have settled these cells break up more quickly than normal cells. The action of lead is therefore double: (1) it increases cellular resistance to reduced osmotic tension, and (2) increases the rate of hemolysis of the cells. These effects have now been observed as constant findings in the blood of thirty-five different individuals, nearly all normal, whose blood belongs in the various isoagglutinating groups. In eight experiments, in which many different concentrations of lead were tried, all results were similar, and there was a definite quantitative relationship between the amount of lead used and the extent of its effect. Three of these eight bloods were obtained from patients suffering from plumbism. The maintenance of a similar quantitative relationship in all cases indicates that the chemical reaction is uninfluenced by immunity or susceptibility. Since the reaction is, therefore, quantitative, a fairly accurate prediction may be made as to the degree a given quantity of lead will influence like numbers of red cells. This is also true of cases where the reticulated count was high—as in a case of lead poisoning with 16 per cent reticulated cells. In the blood of two cases of hemolytic jaundice in which the reticulated cell count was as high as 26 per cent and 16 per cent, and in which hemolysis took place with abnormal ease, we observed that the difference between the curves for control and "leaded" cells was approximately that found in experiments with normal blood. It is therefore probable that this phenomenon appears constantly in human red cells washed free from plasma, and gives no indication of susceptibility to lead intoxication or lead anemia.

1. The Quantitative and Specific Resistance to Reduced Osmotic Tension.—The most striking results which were found in the first series of experiments showed that after exposure to 0.01 mg. of Pb per

cc. (1 part per 100,000) the power of red blood cells to resist lowered osmotic tension was definitely increased, and that they did not hemolyze within 18 hours. This concentration was therefore employed in all further experiments, even though it did not exercise a maximal effect on the resistance.

If the results of a series of ten such experiments are plotted in a distribution chart (Text-fig. 2) in which each point represents the



TEXT-FIG. 2. The distribution of hemolysis. The average of ten experiments with cells exposed to 0.01 mg. of lead as lead chloride per cc. of washed red blood cells for 1 hour. Each point represents the per cent increase of hemolysis over that in the next tube of greater saline concentration.

●———● Normal cells. x– – – –x "Leaded" cells.

increase in per cent of cells hemolyzed, it appears clearly that the cells are unevenly "leaded," or that the phenomenon involves two reactions, one of which permits hemolysis in concentrations above 0.225 per cent salt solution, while the other prevents hemolysis until the concentration of salt falls to 0.100 per cent.

In another series of twenty consecutive experiments an average of only 69 per cent of all the "leaded" cells hemolyzed at this saline dilution.[3] The invariable movement toward the left of the curve representing hemolysis of "leaded" cells suggests that the normal cells which hemolyze most easily are those which are most susceptible to lead and are made more resistant by its action. Just which cells these may be—reticulated or mature, young or old—has not yet been definitely demonstrated (20); but the chemical reaction which probably causes the change in hemolysis will be discussed in the third paper of this series.

Attempts to reverse this reaction, and to "de-lead" the red cells proved unsuccessful. They consisted in soaking "leaded" cells in isotonic solutions of sodium acetate, sodium glycerophosphate, and sodium tartrate,[4] and then determining their resistance to hypotonic saline solution. The results of this procedure, as well as those obtained from repeated washing of cells in Ringer solution, indicate that the lead reaction is irreversible with such substances as might dissolve a lead precipitate without destroying the blood cells.

The next problem investigated was the effect of various lead salts and other metals. From Table I it is clear that all the soluble lead salts studied affected red blood cells similarly, while the salts of other metals showed no comparable effect after exposures of 1 hour. As already demonstrated by Gunn (21), Hill (22), and Greenthal and O'Donnell (23), arsenic shows some effect, but even with such a relatively high concentration as 1.5 mg. per cc. this is very slight in comparison with that of lead. Mercury, likewise, affects the cells as Detre and Sellei (24) first demonstrated, but only at the high concentration of 1 per cent, when it "fixes" the cells by precipitation of their proteins. A different mechanism apparently causes hemolysis after exposure to mercuric chloride. It is therefore probable that the action of lead on the resistance of red blood cells to hypotonic salt solution is an isolated phenomenon which appears only with the various lead salts which were tested, at least under the conditions and in the concentrations of these experiments. It is not evidence of a

[3] These were not included in the text-figure because 0.100 per cent saline was the lowest dilution tested, and a complete curve was therefore not obtained.

[4] This caused considerable hemolysis of both normal and "leaded" cells.

TABLE I.

Hemolysis of Red Blood Cells in Hypotonic Saline after Their Exposure to Various Substances.

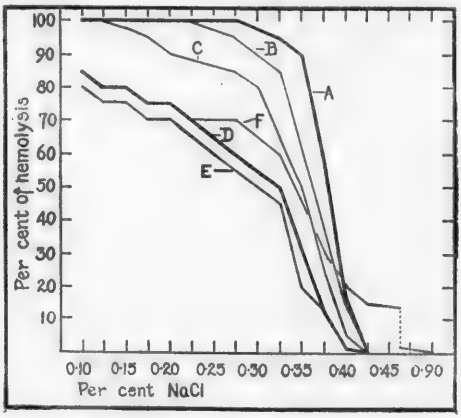
Substance used.	No. of experiments.	Concentration of substance per cc. of red blood cells.	Per cent concentration of saline.				Remarks.
			Start of hemolysis.		Completion of hemolysis.		
			Control cells.	Test cells.	Control cells.	Test cells.	
		mg.					
Pb as acetate.	16	0.025	0.550	0.550	0.300	0.300	Slight effect between 0.300 and 0.550.
	16	0.15	0.550	0.400	0.300	0.300*	Marked effect.
	16	0.25	0.550	0.400	0.300	0.300*	" "
Pb as nitrate.	1	0.08	0.500	0.325	0.200	0.100†	" "
Cerium as chloride.	1	0.2	0.400	0.400	0.250	0.250	pH 6.5. No effect except agglutination. With stronger solutions only effect is increased hemolysis of test cells, and agglutination.
	1	0.1					
	1	2.0					
	1	1.0					
Al as chloride.	1	0.004	0.450	0.450	0.275	0.275	pH 3.9. Stronger concentrations caused agglutination and hemolysis.
Ca " "	1	0.36	0.475	0.475	0.225	0.225	pH 5. Increased hemolysis in tubes between 0.225 and 0.475.
As as arsenious acid.	1	1.5	0.425	0.450	0.250	0.200	pH 6.6. Very slight protection down to 0.025. Hemolysis in increased concentrations.
	1	0.15					No effect.
	1	3.00					" "
	1	0.60		0.500		0.200	Very slight effect.
	1	0.75	0.525	0.500	0.200	0.200	Moderate effect between 0.200 and 0.500.
As as sodium arsenite.	1	0.095					No effect.
	1	0.3					
	2	0.08	0.450	0.450	0.200	0.200	Slight effect between 0.200 and 0.450.

As as sodium arsenate.	1	0.09					No effect.
	1	0.20					
	2	0.07	0.450	0.450	0.200	0.200	Slight effect between 0.200 and 0.450.
As as Fowler's solution.	1	0.38	0.525	0.525	0.200	0.200	No effect. Slight increase in hemolysis.
	2	0.07	0.450	0.450	0.200	0.200	" "
Cd as chloride.	2	0.4	0.525	0.525	0.200	0.200	" "
Cu " "	1	3.0	0.500	0.500	0.225	0.300	No effect except slight hemolysis.
Hg as mercuric chloride.	1	0.02	0.450	0.900‡	0.200	0.200	Marked hemolysis.
	1	0.001	0.450	0.450	0.200	0.100	Very slight effect.
	1	14.8	0.450	0.900§	0.200	0.100‖	Hemoglobin precipitated.
Serum.	1	0.04 cc.	0.450	0.450	0.250	0.250	Slightly less hemolysis at 0.400.
Formalin.	1	0.4%	0.450	0.450	0.200	0.100	Slight effect.
	1	0.04%	0.450	0.450	0.200	0.100	Very slight effect. Greater concentrations cause hemolysis.
	1	0.02%	0.500	0.500	0.225	0.225	No effect.
	1	0.002%	0.500	0.500	0.225	0.225	" "
Tannic acid.	1	0.0002%					" "
	1	0.02%	0.400	0.400	0.200	0.200	" "
	1	0.008%	0.400	0.400	0.200	0.200	" "
Sodium tannate.	1	0.008%	0.400	0.400	0.200	0.200	" "

* Below 0.300.
† " 0.100.
‡ Hemolysis in 0.900.
§ 20 per cent hemolysis in 0.900
‖ 20 " " " " 0.100

general reaction with metals, or merely of a given valency or ionization, for if these were the governing influences, then other substances—particularly trivalent metals like aluminum—would cause a similar reaction.

2. Effect of Time, Temperature, and Diffusion.—If this reaction is chemical it is to be expected that the establishment of equilibrium would take time, and that a high temperature would increase its



TEXT-FIG. 3. Sodium chloride hemolysis. Effect of varying the time of exposure to lead. The curves show the effect on the hemolysis of washed red blood cells of previous exposure to:

B, 1 cc. of red blood cells exposed for 15 min. to 0.01 mg. of Pb as chloride.
C, 1 " " " " " " " 30 " " 0.01 " " " " "
D, 1 " " " " " " " 1 hr. " 0.01 " " " " "
E, 1 " " " " " " " 3 hrs. " 0.01 " " " " "
F, 1 " " " " " " " 9 " " 0.01 " " " " "
A shows the action of the control normal cells in Ringer solution.

speed. This proved to be true. Text-fig. 3, which represents one of three similar experiments, shows that the reaction is very incomplete after 30 minutes but nearly complete after 1 hour exposure to lead at room temperature. Observations after exposures lasting 5 and 7 hours, which are not shown in the chart, demonstrate the tendency to rapid destruction and hemolysis even in high concentrations of salt. In the observations made after 9 hours exposure this reaction was slightly increased. This experiment is important, for it is added

evidence for the short life of "leaded" cells. It demonstrates that their resistance to decreased osmotic tension gradually increases, and that later there is increased hemolysis of some cells even in higher concentrations of salt. This rapid change in reaction does not occur with the normal cells of the control.

Another experiment shows the relationship between concentration of lead and time. Three concentrations were used (Table II) and the rate of reaction was noted at intervals. Those cells exposed to the highest concentration of lead (0.08 mg. of Pb per cc.) reached their maximum "fixation" or greatest resistance in 25 minutes. All tubes when tested at the end of 2 hours showed a definite increase of hemolysis. Cells exposed to 0.04 mg. of lead reached maximum "fixation" after 1 and 2 hours and at that time showed signs of hemolysis. Those

TABLE II.

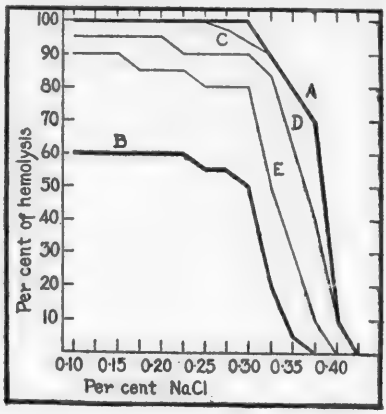
Fixation in 0.300 Per Cent NaCl after Exposure to Lead Chloride.

Concentration of lead chloride per cc.	Per cent hemolysis after exposure for			
	½ hr.	1 hr.	2 hrs.	6 hrs.
mg.				
Control.	100	100	100	100
0.08	20	15	30	50
0.04	35	8	5	18
0.008	100	30	12	10

exposed to 0.008 mg. of Pb per cc. manifested progressively increasing degrees of "fixation" up to 2 hours. When examined after 6 hours exposure to lead they still showed maximal "fixation" without evidence of general hemolysis. One experiment demonstrated the occurrence of a slight acceleration of these phenomena when the cells were kept at 30°C. instead of at room temperature.

3. Evidences of this Reaction in Vivo.—The questions which at once arise are whether this phenomenon can occur in the presence of blood plasma, and whether it occurs in life. Two types of experiments were performed to determine whether normal plasma neutralizes the lead and prevents its action on red blood cells. In the first of these, the solution of lead is mixed with serum before being added to the red corpuscles; in the second, a solution of lead is mixed directly with

whole blood. When lead and serum are first united and allowed to stand at room temperature for 30 minutes, the serum neutralizes the lead completely. In a series of twenty-two experiments—in six of which the subjects had lead poisoning—the combining power of serum and lead was found to be surprisingly constant. Generally 0.022 to 0.035 cc. of blood serum counteracted the effect of 0.01 mg. of lead upon 1 cc. of red blood corpuscles; smaller quantities of serum



TEXT-FIG. 4. Sodium chloride hemolysis. Effect of a constant quantum of lead and varying quantities of blood serum.

A, control of normal red blood cells in Ringer solution.

B, 1 cc. of red blood cells exposed for 1 hr. to 0.01 mg. of Pb as chloride.

C, 1 " " " " " " 1 " " 0.01 " " Pb and 0.05 cc. of blood serum.

D, 1 cc. of red blood cells exposed for 1 hr. to 0.01 mg. of Pb and 0.03 cc. of blood serum.

E, 1 cc. of red blood cells exposed for 1 hr. to 0.01 mg. of Pb and 0.02 cc. of blood serum.

eliminated only part of the lead. Text-fig. 4 illustrates a typical experiment and demonstrates that some constituent in the serum may so react with the lead salt that it no longer affects the red corpuscles.[5]

Similar evidence for the chemical binding of lead is obtained by mixing red blood cells with the lead solution. After standing for 30 minutes this mixture is centrifuged and the supernatant fluid pipetted

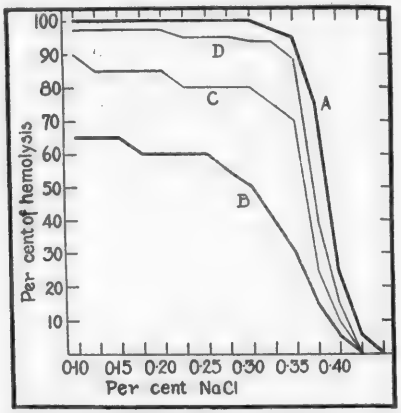
[5] The mechanism of this reaction is discussed in Part 3 of this series.

off. The relative volumes are so arranged that the supernatant fluid should then contain 0.01 mg. of lead per cc. When other red cells are exposed to this, however, no evidence of the effect of lead can be found. This again shows that the lead has been bound by some constituent of the red cells. Hemolyzed red cells have a similar action. Two experiments showed that if a given volume of cells is laked in distilled water, to which double strength Ringer solution is later added to bring the final concentration of salt to 0.9 per cent, their power to neutralize lead is the same as that of an equal volume of intact cells. It is interesting to note that the red blood cells from a unit volume of blood have approximately the same power to combine with lead as has the serum from the same unit quantity.

When lead is added to defibrinated whole blood, however, the serum does not completely inhibit its reaction with the blood cells. In four such experiments it was clear that part of the lead united with the red blood cells, although it was necessary to add more than four times the usual amount of lead salt to cause an effect comparable to that in washed cells. Text-fig. 5 shows figures from a typical experiment of this series. The apparent interpretation depends upon the fact that the reaction with lead may occur simultaneously with serum and cells, and that both are therefore involved.

Diffusion of substances out of the red cells is also important in the reaction with lead. This was tested by allowing washed red cells to lie in Ringer solution for varying lengths of time, and then centrifuging them once before exposure to lead. In five out of six experiments the usual solution of lead (0.01 mg. per cc.) produced no alteration in the normal resistance to hypotonic solutions of cells which had been in Ringer solution for 6 hours. More concentrated solutions (0.1 mg. of Pb per cc.) however, still increased the cellular resistance. After the saline solution surrounding these cells was thoroughly removed by repeated washing and centrifuging, the red cells reacted as before with the usual low concentrations of lead. It is, therefore, evident that something which usually reacts with the lead has diffused from the cells into the surrounding media, and has there reacted with the lead. These experiments are more fully outlined in the third paper of this series (page 194). They are reported here merely to indicate further evidence of a chemical explanation of the action of lead on the blood.

If this reaction between lead and red corpuscles has any great significance its occurrence in life must, of course, be demonstrable. That it is, has already been shown in man by von Liebermann (16), by Orbán (17), and by Hayhurst (18), who found that corpuscular resistance increased in acute lead poisoning. However, the resistance of corpuscles was further tested in this laboratory in six rabbits before and after the ingestion of 1 gm. of lead acetate. Five of these animals showed similar reactions to the lead, the sixth showed no change either in resistance of the red cells, in anemia, or in stippling. Text-

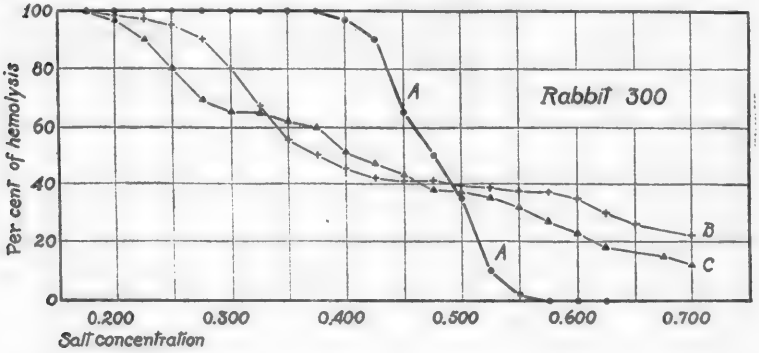


TEXT-FIG. 5. Sodium chloride hemolysis. Effect of lead added to whole blood.
A, control of red blood cells washed in Ringer solution.
B, 1 cc. of washed red blood cells exposed to 0.01 mg. of Pb as chloride.
C, 1 " " whole blood exposed to 0.04 mg. of Pb as chloride.
D, 1 " " " " " " 0.01 " " " " "

fig. 6 shows the characteristic, though most marked effect obtained. This rabbit had suffered from a secondary anemia due to repeated bleeding prior to the ingestion of lead (see Key (3)), and it died with a marked anemia 2 days after lead was given. The chart demonstrates that the corpuscles are affected just as *in vitro*, that many resist the strain of hypotonic saline better than the control cells, but that many hemolyze quickly even in normal saline solution. These experiments justify the assumption that the phenomena studied here *in vitro* depict reactions similar to those which may occur in life. They

are most probably related to development of the anemia of lead poisoning. This is further indicated by the fact that the effect of lead on hemolysis in hypotonic solutions seems to run parallel to the appearance of stippling and anemia which occurs in different species of animals during life.

These phenomena are not found in all species, as can be seen in Table III, but in the species studied all or none have been observed. The results of the *in vitro* experiment, therefore, run parallel to those found after lead has been allowed to act *in vivo*. Thus, stippling and anemia are easily produced in man, rabbits, guinea pigs, and rats; and diminished hemolysis in hypotonic salt solution is also marked.



TEXT-FIG. 6. Sodium chloride hemolysis. The effect of acute lead poisoning on red blood cells *in vivo*. The curves represent the action of red blood cells washed in Ringer solution.

A, before lead was given.

B, 19 hours after 1 gm. of lead acetate was given by mouth.

C, 43 " " 1 " " " " " " " " "

The red cells of horses, dogs, and cats do not hemolyze after exposure to these dilute solutions of lead; and, although for some species the data here presented are incomplete, it is clear that the cat shows no symptoms of anemia until the end of the disease, and that no definite stippling has been observed in its blood.[6] The causes for this variation among different species are not yet clear.

[6] Occasional granulation of the red cells was seen, but this was indefinite in outline, did not stain darkly, and was rare. This is similar to the experience of Nehring (25).

In two experiments tests were made to determine whether lead reacts with cat blood even though there is no change in resistance to hypotonic salt solutions. These tests were made by adding 0.1 mg. of lead in 1 cc. of Ringer solution to 5 cc. of thoroughly washed red cells from cat blood. After standing for an hour this mixture was centrifuged and the supernatant fluid pipetted off. If no reaction

TABLE III.

The Parallelism between Blood Manifestations in Various Animal Species. The Effect of Lead in Vivo on the Appearance of Anemia and Stippling in Blood, and in Vitro, on the Hemolysis in Hypotonic Salt Solution.

Species.	Distortion of hypotonic saline curve to left following exposure to lead.*	Appearance of anemia in "leaded" animals.†	Appearance of stippling in "leaded" animals.†
Man	++	+	+
Rabbit	++	+	+
Rat	+++	+	+
Guinea pig	+	+‡	+‡
Dog	Negative.	?	?§
Cat	"	Negative until just before death.	No clear cut stippling. Rare mottling of cells.
Horse	"	?	?
Chicken	"	?	Negative.‖

* All exposed to same concentration, *i.e.* 0.01 mg. of Pb per cc. of washed red blood cells. Plus means that the curve of "leaded" cells indicated distinctly increased resistance to hemolysis in dilute saline solutions.

† Concentrations of Pb per kg. of animal not constant.

‡ Data from Sabrazès, Bourret, and Léger (26), and from Beresina and Engling (26).

§ Stated by White and Pepper (28) to be positive, though not typical. This observation has not been repeated.

‖ Data from Key (3) and also from Meyer and Speroni (27).

had taken place the supernatant fluid should react with washed red cells from man, evidence for the reaction being found by testing with hypotonic salt solutions. 1.5 cc. of the supernatant fluid (*i.e.* 0.025 mg. of lead), were, therefore, added to 2.5 cc. of washed human red corpuscles. No change in hemolysis in hypotonic salt solution resulted. Chemical reaction had, therefore, taken place with the cat corpuscles, but without affecting their resistance to hypotonic

salt solutions. The results of *in vitro* experiments, therefore, are parallel to those obtained with lead *in vivo*.

4. The Effect of Lead on the Length of Life of the Red Cells.—In Text-figs. 1 and 4 two phenomena of red cells can be seen (1) the increase in their resistance to lowered osmotic tension, and (2) the increased rate of destruction of blood even in normal salt solution. This latter phenomenon is striking when red corpuscles are exposed to higher concentrations of lead. But in the following experiments such a concentration was used (0.01 mg. of Pb per cc.) that there was no more hemolysis after standing for several hours in normal saline, than was found in control cells. After being treated with lead the corpuscles were put in one tonometer, and normal washed red blood cells in another. The controls were treated in every way exactly like "leaded" cells, except for exposure to normal salt solution instead of to normal salt solution plus lead. All manipulations were carried out simultaneously in the same apparatus. Neither control nor "leaded" cells hemolyzed markedly before they were put in the tonometers. Both groups of cells should, theoretically, resist rotation equally well, but when the suspensions were centrifuged, after being slowly rotated for 5 minutes, hemolysis, due to the rolling of the corpuscles on glass, was markedly increased in the lead suspensions and absent in the controls (nine experiments). Shaking the tonometers in a shaking machine gave similar results (one experiment).

In another group of experiments we exposed cells intermittently to atmospheres rich in CO_2, or to alveolar air, as well as to room air, because it was thought that perhaps the gaseous exchange, by changing the volume of the corpuscles (29), would cause greater destruction of "leaded" cells. The technique used was as follows:

Washed red blood cells were put into four tonometers, two containing "leaded" cells, and two, normal washed cells. The cell counts of the blood in all tubes in each experiment were similar. Air was slowly blown through the control tubes while an equal amount of gas rich in CO_2 was passed through the others. Then, at a constant temperature of 37° or 25° all four were slowly rotated in the same machine for a short while. After blowing air through all four tonometers for a given period, rotation was again begun. This whole procedure was repeated three times. Since the cell counts in all tubes agreed closely, any differences in degree of final hemolysis must have been due to the action of lead on the cells or to the gas mixtures, which were the only variables in the experiments. All manip-

ulations during centrifugalization and rotation were performed very carefully to prevent all possible trauma.

After the rotation necessary to establish equilibrium, all the "leaded" solutions showed greater hemolysis than the controls. In seven of the nine experiments the exposure to high concentrations of CO_2 did not cause a greater percentage increase of hemolysis in the tubes containing lead than in the saline controls. Perhaps more frequent exposure to CO_2 might cause greater differentiation, but no experiments to test this were performed because of the marked hemolysis in "leaded" cells following slight rotation alone. Hemolysis of both normal and "leaded" cells was always more complete after alternate exposure to CO_2 and room air, than after exposure to room air only.

In the last three experiments the per cent of hemolysis of the different samples of blood was:

Hemolysis of Red Blood Cells Produced by Swelling and Shrinking, and by Rotation.

Exp. No.	Type of cells.	Amount of lead per cc. of defibrinated blood.	Analysis of CO_2-rich gas mixture.	Duration of total rotation.	Per cent of hemolysis in blood.		
					Before rotation or exposure to CO_2.	After rotation and exposure to room air alone.	After rotation in atmospheres of CO_2 and room air.
		mg.		*min.*			
162	Normal.		2% O_2	15		0.5	2.0
	"Leaded."	0.2	8.5% CO_2	15		5.0	9.0
190	Normal.			9	0.25	0.5	1.5
	"Leaded."	0.2	Pure CO_2	9	10.00	12.0	16.0
191	Normal.			6	0.0	0.0	2.0
	"Leaded."	0.05	Pure CO_2	6	0.5	1.7	2.5

These figures were checked in Experiment 162 by determination of the oxygen-combining power of the supernatant fluid. This method gave approximately the same results as did the colorimetric test.

Such experiments indicate that slight trauma destroys "leaded" cells far more readily than normal cells, but give no good evidence that the change in volume which occurs in each cycle of the circulation, causes a greater destruction of "leaded" red corpuscles than of normal

cells. Rous and Robertson (30) and recently Broun (31) have attributed destruction of the blood to mechanical trauma; and these experiments provide an illustration of a state in which cells injured by lead would be very much more fragile than normal and, therefore, far more susceptible to the traumata involved in circulation. This is probably a very important factor in the increased destruction of blood in plumbism.

The Effect of Lead on Other Forms of Hemolysis.

The effect of lead upon other types of hemolysis was also tried. Five experiments were carried out with saponin hemolysis. Three of these showed a similar though far less marked effect than that seen in the hypotonic salt test. The "leaded" cells hemolyzed less readily than did the control cells. The other two experiments were essentially negative. These results are not striking enough to be much emphasized, and give but little new indication of the reactions involved in the phenomenon. Even though saponin probably acts by dissolving the lipoids of the cells, these experiments do not necessarily indicate that the action of lead is upon these lipoids, as any other effect upon the cell membrane might well distort the effect of saponin.

The effect of anti-human serum was also tried on "leaded" cells.[7] Three experiments were done with the serum of a rabbit which had been immunized against human blood. The amboceptor was diluted 1 to 15, 25, and 40 in the different experiments. In the three experiments the "leaded" cells showed more rapid and complete hemolysis than did the control cells. The significance of this is not yet clear, but the data may be important in the explanation of mechanisms of various reactions which occur in hemolysis.

CONCLUSIONS.

It appears, from the investigations in other laboratories, that the anemia observed in cases of lead poisoning is due to destruction of blood rather than to diminished production of blood. The method of poisoning cells *in vitro* with lead was adopted in order to study this

[7] These experiments were made possible by the cooperation of Dr. William A. Hinton. We take this opportunity to thank him for furnishing the immune serum and for his help in the experiments.

phenomenon, and distinct effects were thereby obtained, even when only 0.001 mg. of lead is added to approximately 5 billion washed red corpuscles. In order to obtain optimum results the usual dosage employed was ten times this or 0.01 mg. per 5 billion cells. The following changes were observed in cells so treated.

1. Such a marked increase in the resistance to hypotonic salt solution develops that complete hemolysis does not occur until the cells are exposed to a saline solution of 0.05 per cent. Untreated cells are completely hemolyzed in 0.25 or 0.225 per cent saline.

2. This reaction is quantitative and varies with the concentration of lead used. Under the conditions of our experiments this phenomenon seems to be unique. The effects of arsenic are very slight in comparison.

3. While from this reaction it may be concluded that lead increases cellular resistance, it also appears that it shortens the life of blood cells. This may be demonstrated by the much more rapid appearance of hemolysis than normal when the cells are merely allowed to stand in Ringer solution of any dilution.

4. In rabbits with acute lead poisoning these same phenomena may be noted *in vivo*.

5. Both phenomena may be changed *in vitro* by varying the time and temperature of the reaction and the concentration of lead, as Fici has already pointed out.

6. If normal cells stand in Ringer solution for 6 hours something diffuses into the solution which largely reduces the action of lead. After repeated washing these cells react with lead in the usual manner.

7. Small amounts of serum react with lead and eliminate its effects. Red blood cells, treated with a mixture of lead and blood serum, show normal hemolysis in hypotonic salt solution.

8. When lead is added to whole blood the only evidence of this neutralization is a marked decrease in the intensity of the reaction. This is probably explained by the fact that since both serum and cells are present together, the lead reacts with both simultaneously. This in turn explains how the reaction may occur *in vivo*.

9. The change in hemolysis does not appear in the blood of all species of animals. In the species in which it does occur, anemia and stippling of the red cells also tend to develop readily during lead intoxication.

10. *In vitro*, cells which have been exposed to lead are far more fragile than normal blood cells. Slight trauma causes them to hemolyze. *In vivo* this is probably an important causal factor in the increased destruction of blood and in the anemia of acute lead poisoning.

11. The serum of a rabbit which has been immunized to human blood, causes more rapid and complete hemolysis in cells treated with lead than in the control cells. No satisfactory explanation for this has been found.

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LEAD STUDIES.

III. The Effects of Lead on Red Blood Cells.

Part 2. Surface Phenomena and Their Physiological Explanation.

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Plate 5.

(Received for publication, March 27, 1924.)

The first paper of this series describes the effects of lead upon the hemolysis of red blood cells. These were so striking that the possibility of demonstrating other changes in red corpuscles was sufficient to justify further experimentation. This paper outlines the positive results of such work. Since in all these studies the mechanisms underlying the phenomena are of great interest we are also recording experiments which help to explain physiologically some of the facts observed.

Agglutination.—The effect of lead on the agglutination of blood corpuscles was studied first. The technique used was as follows:

Red cells from human blood belonging to either Group II or Group III were washed three times with Ringer solution (pH 6.5). Equal parts of this cell suspension were then exposed to varying concentrations of lead in Ringer solution and to Ringer solution alone, and, after 1 hour, were again washed with Ringer solution. The cells were then mixed with Group IV serum in a white counter as Ashby describes (1). In individual experiments the concentration of corpuscles in all tubes was maintained approximately the same.

Six such experiments consistently showed that, after exposure to lead, agglutination was slow and slight if it occurred at all, whereas, in the control tubes, exposed merely to Ringer solution, strong agglutina-

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tion followed the addition of serum. Table I shows the number of unagglutinated cells in several experiments. The difficulty of accurately counting unagglutinated cells increases progressively as agglutination decreases, for no cells which touch even one other cell may be considered free. Such contact, of course, although it may be purely accidental, lowers the count markedly.

The photomicrographs demonstrate even more strikingly the degree to which these small quantities of lead inhibit agglutination.

Stickiness of Red Blood Corpuscles.—The discovery that lead inhibits agglutination of red blood cells in heterologous serum naturally makes one question whether it affects the natural stickiness of the cell

TABLE I.

Effect of Lead on the Agglutination of Red Blood Cells. Group II Blood Cells Exposed to Group IV Serum.

Subject.	No. of cells per c. mm. remaining unagglutinated after exposure to serum.				
	Normal control cells.	Cells treated with lead before exposure to serum.			
		Concentrations of lead per cc. of red blood cells.			
		0.01 mg.	0.03 mg.	0.06 mg.	0.1 mg.
P. D.	26,400	61,100	121,000	124,000	105,000
	44,000	101,000	103,000	108,000	257,000
L. F.	12,700	123,000	210,000		293,000
M. O.	69,300	163,000	196,000	294,000	
G. O.	112,000	212,000		424,000	

surface or whether the addition of agglutinating serum is the cause of such a change. This problem was studied by means of Fenn's method (2) which was followed in every detail.

In brief this method consists of placing a suspension of cells in a glass chamber which is later rotated 180°. The number of cells which remains on the ceiling of the chamber, as well as the number which falls off, is then determined under the microscope. In the preliminary experiments all cells, whether normal or treated with lead, stuck to the ceiling; but later it was found that a clear differentiation could be made by adding to the Ringer solution a small amount of blood serum (0.6 cc. per 100 cc. of Ringer solution). A hydrogen ion concentration of 6.4 was maintained in all solutions. Before rotation, all cells except the controls were exposed to lead chloride (concentrations of 0.03 and 0.05 mg. of Pb per cc.)

for 1 hour, and were then washed once with an excess of Ringer solution. All manipulations of cells treated with lead were, of course, repeated on the control cells with Ringer solution alone.

In the five experiments performed, all results agreed. Practically all the normal control cells remained on the ceiling of the chamber after rotation; whereas a large proportion of "leaded" cells fell (see Table II). From this it may be concluded that, after exposure to lead, the surface of red corpuscles is less sticky than normal. There are two explanations for this; the charge of the cell surface may have changed; or, because of alterations in its chemical composition, the surface may have changed its physical characteristics (Part 3).

To study further this latter suggestion the action of lead on polymorphonuclear leucocytes was tested because their surface is physiologically different from that of red cells.

TABLE II.

Effect of Lead on Normal Stickiness of Red Blood Cells. Adhesion of Cells to Ceiling of Glass Container.

Normal cells.			"Leaded" cells.*		
No. adherent.	No. detached.	Per cent detached.	No. adherent.	No. detached.	Per cent detached.
355	19	5	105	200	66
210	15	7	70	158	69
335	24	7	120	77	39
All.	0	0	34	45	57
"	0	0	175	202	54

* 0.05 mg. of Pb per cc. of red blood cells.

They were obtained from the peritoneal cavity of rats after injection of aleuronat. These were then washed in Ringer solution containing 0.1 per cent of sodium citrate—a procedure which Fenn advised—and then in Ringer solution alone. The concentration of lead in the solutions to which they were then exposed varied from 0.0 to 0.15 mg. per cc., and the concentration of serum in the solutions surrounding leucocytes in the glass chamber was graduated from 0 to 20 per cent of the mixture.

Eight experiments showed that practically all the cells stuck to the ceiling, and that no definite difference between the stickiness of "leaded" white cells and that of normal controls could be observed.

It therefore seems clear that lead affects white and red cells differently. This fact was also suggested by the work of Fine (3), which, in connection with observations reported in Part 1, indicates that lead is less toxic for white cells than for red, particularly when administered in relatively large quantities.

The following experiments were performed in the hope of finding a physiological explanation of these various phenomena, particularly the increased resistance of "leaded" red cells to hypotonic saline (Part 1).

Permeability of the Cell Surface.—In the salt hemolysis test the mechanism studied was the permeability of the cell to water, and the elasticity of its surface. When osmotic tensions of surrounding fluids vary, the cell swells or shrinks because of changes in water content. Only the elasticity of the cell will determine the degree of change of osmotic pressure which the cell can withstand without hemolysis. The permeability to water of normal cells and those exposed to lead was therefore studied in order to determine whether any change in elasticity occurred.

Two methods were employed:

1. The specific gravity of red blood cells was determined. With a definite content of solids, variations in the specific gravity should represent largely a transportation of water in or out of the cell, which should be largely dependent upon the permeability of the surface, or upon the elasticity of the membrane. Clearly, if the membrane were inelastic the entrance of water into the cell would be prevented in spite of normal permeability. Of course, an increased permeability accompanied by an abnormal escape of the solids of "leaded" cells into the bathing solutions would introduce a possible error in such experiments, but the occurrence of this is improbable because the specific gravity of "leaded" cells is higher than normal (Table III). If solid constituents diffused from the cells their specific gravity should be reduced. Determinations made by the second more direct method also show that this error is improbable.

2. The volume of a unit number of cells was determined by means of simultaneous hematocrit readings and cell counts.

These two methods, when used upon cells exposed to different

osmotic tensions, have allowed us to determine with some degree of precision the relative volume of a unit number of cells and the ability of normal and "leaded" cells to shrink and swell.

1. The Effect of Lead on the Specific Gravity of Blood Cells.—The specific gravity of red blood corpuscles was determined by the suspension method described by Reznikoff (4).

Three types of experiments were performed by this method in order to determine whether the specific gravity of "leaded" as well as of

TABLE III.

The Specific Gravity of Red Blood Cells.

Normal cells.	"Leaded" cells.
1.094	1.104
1.088	1.113
1.089	1.111
1.089	1.106
1.096	1.107
Average.....1.091	1.108

TABLE IV.

The Specific Gravity of Red Blood Cells after Exposure to Lead for Varying Lengths of Time.

Time.	Specific gravity.
15 min.	1.089
30 "	1.098
1 hr.	1.115
2 hrs.	1.113
3 "	1.112

normal cells changes when the osmotic tension of the medium surrounding them is varied. From such observations we may discover whether or not water and, perhaps, salts can pass normally through the membrane of "leaded" cells.

Five experiments showed that after treatment with lead the specific gravity of washed red blood cells in normal Ringer solution is always distinctly higher than that of the normal cells (Table III). This difference is so great as to be well outside the limits of experimental

error. The change, as would be expected, develops slowly and reaches its maximum only after an hour (Table IV). Since this change may be observed after the addition of as small a quantity of lead as 0.01 mg. per cc. it cannot be explained by the mere chemical effect of adding the heavy metal; but must be due to the fact that corpuscles decrease in volume after exposure to lead, although otherwise the osmotic tension of the surrounding fluid is not altered.

In two experiments in which the osmotic tension of the suspension fluid was varied, it was found that the specific gravity of both normal and "leaded" cells also changed. The technique used for preparing

TABLE V.

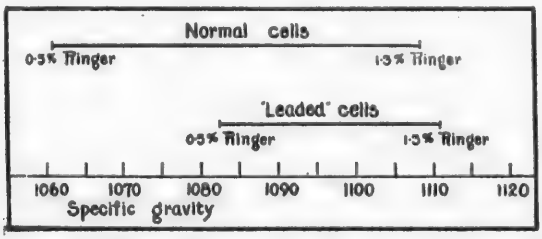
Effect of Osmotic Changes on Specific Gravity.

Concentration of Ringer solution.		Specific gravity of		Change of specific gravity from cells in normal Ringer solution.		Total change of specific gravity.	
Before and during "leading."	After "leading."	Normal cells.	"Leaded" cells.	Normal cells.	"Leaded" cells.	Normal cells.	"Leaded" cells.
0.9	0.9	1.089	1.111				
0.9	0.5	1.065	1.103	−24	−8		
0.9	1.35	1.109	1.115	+20	+4		
0.5	0.9	1.092	1.113	+3	+2		
1.35	0.9	1.090	1.114	+1	+3		
0.9	0.9	1.089	1.106				
0.5	0.5	1.069	1.097	−20	−9		
1.35	1.35	1.104	1.110	+15	+4		
0.5	1.35	1.107	1.115	+18	+9	+38	+18
1.35	0.5	1.073	1.095	−16	−11	−31	−15

the blood cells was similar in every way to that of the previous group except that in one experiment 0.04 mg. of Pb per cc. was added to whole blood, but the results in this experiment agreed with those obtained with washed red cells. After exposure to lead for 3 hours at 30°C. in normal Ringer solution, the suspension of corpuscles was thoroughly centrifuged. Equal parts of the cellular sediment were then added to equal volumes of Ringer solution of varying concentration (0.5, 0.9, and 1.35 per cent). At the end of 20 minutes the specific gravity of the corpuscles in the different tubes was determined (first experiment in Table V). Results showed that after treatment

with lead, red blood cells respond to osmotic changes in the media surrounding them with far less shrinking and swelling than do the normal cells used as control.

The third group of experiments (four in number) demonstrates the degree to which "leaded" and normal cells may shrink and swell if the concentration of the medium surrounding them is varied before the addition of lead. In some cases the cells were first washed with 0.5 per cent Ringer solution, which caused swelling. They were then exposed to lead, in the form of lead chloride, in 0.5 per cent Ringer solution, for 3 hours. The change in specific gravity which occurred after washing with 0.9 or 1.3 per cent Ringer solution was



TEXT-FIG. 1. Experiment demonstrating total changes of specific gravity of cells in varying concentrations of Ringer solution.

then compared with that found with normal control cells. In other tubes lead was added in 1.3 per cent of Ringer solution, and washing took place in concentrations of 0.9 or 0.5 per cent. Text-fig. 1 and the second experiment of Table V show typical results obtained in two such experiments. "Leaded" cells proved to be heavier in every case and their range of swelling and shrinking far smaller than normal. But they are not impervious to water for variations in weight within these restricted limits may be observed; and their usual specific gravity is regained in 0.9 per cent Ringer solution even after exposure to lead in solutions of other concentrations. The difference observed is apparently due to the fact that the exchange of substances through the cellular membrane is restricted—the permeability to water and possibly also to salts is apparently present, but quantitatively decreased. This is best explained by assuming that probably the cell

shrinks and becomes less elastic, or that possibly there is a change in the charge of the cell surface which would repel a definite quantity of water (page 203).

This diminished ability of the corpuscles to swell is probably important in explaining their greater resistance to hypotonic salt solution after exposure to lead (Part 1); for since the cell surface is not so stretched by swelling it does not break so readily. Within the normal physiological range of swelling in the body, however, the water exchange of "leaded" cells may well be normal, for it would not approach the degree of variation studied in these experiments.

2. Determination of the Volume of a Unit Number of Corpuscles.— The second method used to differentiate between normal and "leaded" cells was based on the assumption that the number of cells in a unit volume would vary with the size of the cells.

Whole blood was centrifuged, the plasma discarded, and similar amounts of Ringer solution, with and without lead chloride (0.04 mg. of Pb per cc.) were added to the cells, and allowed to stand for 3 hours. The concentration of the saline solution around both "leaded" and control cells was then changed from 0.9 to 0.6 per cent by mixing one volume of corpuscles with two volumes of 0.45 per cent Ringer solution. In no case did hemolysis follow this procedure. The red cell count of both normal and "leaded" cells was determined in both the 0.9 and 0.6 per cent solutions, and hematocrit determinations were made. The same hemocytometer squares and the same pipette were used throughout. The hematocrit ran for exactly 10 minutes at a speed of 1,860 revolutions per minute.

From data obtained in this way the number of cells packed into a unit volume and, therefore, the change of cell volume should be determined.

A considerable error in making the blood counts was introduced by dilution. In undiluted blood the maximum deviation from the mean was 5 per cent; in 0.6 per cent Ringer solution duplicate counts differed as much as 20 per cent. It was, therefore, important to compare the actual count obtained with the theoretical (one third of the number of cells found before dilution). Of seventeen experiments only two (Table VI) gave results in which this error did not vitiate the significance of the data. In the first of these, in which each figure is the average of five excellent determinations, the normal cell count in 0.6 per cent Ringer solution varies 7.7 per

TABLE VI.

The Effect of Change in the Osmotic Pressure upon Red Blood Cell Volume.

Experiment No.	Normal red blood cells.						"Leaded" red blood cells.					
	0.9% Ringer solution.			0.6% Ringer solution.			0.9% Ringer solution.			0.6% Ringer solution.		
	Hematocrit.	Red blood cell count.	Red blood cell count per unit volume.	Hematocrit.	Red blood cell count.	Red blood cell count per unit volume.	Hematocrit.	Red blood cell count.	Red blood cell count per unit volume.	Hematocrit.	Red blood cell count.	Red blood cell count per unit volume.
1	37.8	4,649,000	123,000	16.2	1,670,000	103,000	35.9	4,375,000	122,000	12.4	1,495,000	121,000
	Swelling 19%.						Swelling 1.0%					
2	37.5	4,810,000	128,000	15	1,606,000	107,000	33.5	4,756,000	142,000	11.75	1,562,000	133,000.
	Swelling 19.8%.						Swelling 6.6%.					

Hematocrit and red blood counts were first made on the bloods in normal Ringer solution. 1 cc. of this blood was then thoroughly mixed with 2 cc. of 0.45 per cent Ringer solution, and hematocrit readings and blood counts again determined. The unit count is the ratio, red blood cells: hematocrit.

cent from the theoretical count. If, in this case, the theoretical figure is substituted for the actual determination it will appear that normal cells swell 28.4 instead of 19 per cent, whereas "leaded" cells swell only 0.06 per cent. In the second experiment where all figures given are averages of two determinations, both of which agree very closely with the theoretical, normal cells swell 19.8 per cent, and "leaded" cells, 6.6 per cent.

Although the inability of cells to swell, except within narrow limits, after exposure to lead is quite apparent, the inaccuracy of this method makes the result of interest only as a check on similar data obtained by the other method.

The Effect of Swelling and Shrinking of Normal and "Leaded" Red Blood Corpuscles upon Hypotonic Salt Hemolysis.

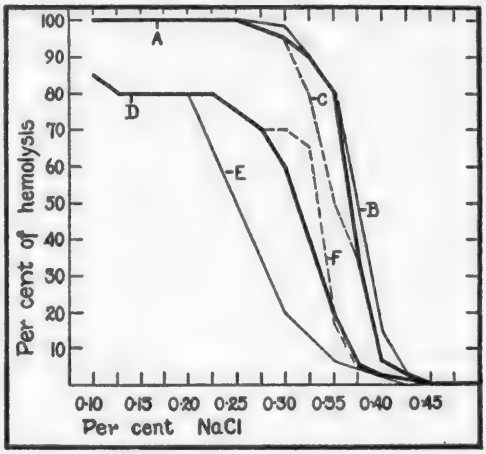
To differentiate more clearly between the swelling and shrinking of normal and "leaded" cells, corpuscles were washed in 0.5, 0.9, and 1.3 per cent solutions of sodium chloride. They were then "leaded" with 0.01 mg. of Pb per cc. of cells in salt solutions which maintained their original osmotic tension. After standing for 1 hour, the control and "leaded" corpuscles were washed once with 0.9 per cent saline solution to establish uniform conditions in all tubes, and the usual hypotonic salt tests were then made.

Four such experiments gave similar results. The data obtained in a typical one of these are graphically recorded in Text-fig. 2. The fragility of the control cells varied only slightly—the greatest hemolysis occurring in those washed originally with 0.5 per cent saline, the least in those washed in 1.3 per cent. The "leaded" cells were more markedly affected and not in the same way, for the cells washed in 0.5 per cent hemolyzed least.

It is hazardous to interpret these facts, but their explanation is possibly to be found in a relative inelasticity and fixation of the cell membrane[1] after exposure to lead. If fixation occurs while the membrane is distended (*i.e.*, in 0.5 per cent Ringer solution), then immersion

[1] "Membrane" as here used means merely a chemical phase, and not necessarily a membranous type of tissue. By changing physical-chemical relationships in such a phase, however, the physiological properties of the cell may be strikingly altered.

in 0.9 per cent Ringer solution and resulting equalization of osmotic tensions should allow the cell to reassume its usual size. The membrane might still, however, be relatively stretched. This should allow the cell to withstand a greater decrease of external tension than when its surface is shrunken, because the first influx of water would cause less internal pressure in the cell of larger surface area, and there-



TEXT-FIG. 2. The effect of swelling and shrinking of normal and "leaded" red blood corpuscles upon hypotonic salt hemolysis.

A, red blood cells in 0.9 per cent saline solution.

B, red blood cells in 0.5 per cent saline and then washed once with 0.9 per cent saline solution.

C, red blood cells in 1.3 per cent saline and then washed once with 0.9 per cent saline solution.

D, red blood cells and Pb 0.01 mg. per cc. in 0.9 per cent saline solution.

E, red blood cells and Pb 0.01 mg. per cc. in 0.5 per cent saline solution and then washed once with 0.9 per cent saline solution.

F, red blood cells and Pb 0.01 mg. per cc. in 1.3 per cent saline and then washed once with 0.9 per cent saline solution.

fore be less apt to cause hemolysis. As endosmosis continues, however, all cells would be exposed to tensions adequate to destroy them. Strength is given to such an hypothesis by the experimental data which show increased resistance of the "swollen" fixed cells only in hypotonic salt dilutions down to 0.225 per cent.

The Appearance of the Cell.—It is to be expected that such changes in the size of corpuscles as are suggested by the specific gravity experiments might be observed under the microscope. By examining large numbers of cells under an oil immersion lens and measuring them with a micrometer eyepiece, definite changes may be observed. If exposed to lead the cell margin first becomes pale and then swelling occurs. Within an hour this is followed by shrinking and definite crenation of the surface; then gradually the irregularity of these shrunk cells is lost and their outline becomes smooth and quite refractile to light. These phenomena, as well as others which also vary with time, suggest that the reaction of the corpuscles with lead is slow and, under the temperature conditions of our experiments, reaches equilibrium within 2 to 5 hours. Meneghetti (5) also found definite change in the appearance of cells exposed to lead salts.

That lead acts upon the surface of red blood cells is suggested by all the preceding data which include (*a*) increased resistance to hypotonic salt solution; (*b*) increased hemolysis on exposure to serum of a rabbit immunized to human blood (Part 1); (*c*) shrinking and accompanying increase in specific gravity; (*d*) inability of the cells to swell sufficiently to equalize large changes in osmotic tension; (*e*) increased rate of hemolysis without manipulation, as observed both *in vitro* and *in vivo* (Part 1); (*f*) disappearance of agglutination; (*g*) loss of normal surface stickiness (observed in red cells and not in white, probably because of their different surface). All these phenomena are good evidence of the surface action of lead. It is, therefore, of interest to determine whether corresponding physiological changes also occur in the interior of red blood corpuscles after exposure to lead. These would be practically dependent upon an interaction between lead and hemoglobin.

The Physiological Effect of Lead on Hemoglobin.—Two types of experiments were employed in studying this question. In the first group lead was mixed with an excess of pure hemoglobin (see Part 3) and the mixture allowed to stand so that any chemical combination might reach equilibrium. If this mixture is then added to suspensions of red blood corpuscles close chemical combinations which may have formed will be demonstrated by the occurrence of a less intense reaction between the lead solution mixture and red cells. By thus using

red blood corpuscles as a quantitative indicator of chemical reactions, it was found that lead, in the concentrations used, does not react with hemoglobin; for the resistance of blood cells to hypotonic salt solution is exactly the same after the addition of the lead-hemoglobin mixture as with the solutions of lead alone. These experiments are more fully described in Part 3.

In the second group of experiments physiological reactions of the red blood cells were observed by carefully studying the effect of lead upon their gas exchange.[2] The CO_2 exchange in reduced and oxygenated blood was found by determining the oxygen and carbon dioxide content of the corpuscles and of the air to which they had been exposed. Except that slightly smaller tonometers were used, the technique was similar to that described by Peters, Barr, and Rule (6). The gas in the tonometers, after the withdrawal of 1 cc. of blood, was analyzed by the Haldane method; blood analyses were made according to Van Slyke's method (7).

The standard procedure was as follows: The serum of approximately 80 cc. of defibrinated normal human blood was separated from the red corpuscles. These cells were then washed; half were exposed to lead in the usual way, and half saved for controls. The number of cells per unit volume was made the same in both suspensions; equal amounts of the serum were then added to the "leaded" and normal cells, thus synthesizing the blood with equal amounts of serum and equal numbers of cells. The carbon dioxide dissociation curves of both "leaded" and control samples were then determined at CO_2 tensions of 20, 40, and 60 mm. of Hg. This experiment was repeated three times with both reduced and oxidized bloods.

The curves thus obtained, demonstrating the reactions of the two bloods, have a similar slope, and those representing the difference of volumes per cent between the oxidized and reduced blood were also similar with both "leaded" and control cells. The oxygen capacity of the "leaded" blood was similar to that of its control; and also at very reduced pressures (6 and 7 mm. O_2) the two blood samples contain almost the same volume per cent of oxygen. No experiments were carried out with intermediate tensions of O_2, but it is reasonable to assume that there is probably no difference between the capacity of normal and "leaded" blood to combine with oxygen because of the

[2] We wish to take this opportunity to thank Dr. Arlie V. Bock for his great help and advice in performing these experiments.

normal behavior of such blood with respect to CO_2, since the close relationship between CO_2 and O_2 is a well established fact. The data are so convincingly negative that no graphic records of the experiments are shown.

These two series of experiments thus demonstrate (*a*) that lead does not react chemically with hemoglobin which is the chief constituent of the interior of the red blood cell and (*b*) that, when studied by present methods, exposure to lead does not alter the normal gas exchange of these cells in spite of the distinct limits of the fluid exchange. From these facts we may readily conclude that the interior of red blood corpuscles is not chemically or physiologically affected by lead.

CONCLUSIONS.

The physiological changes following the reaction of lead upon red blood cells are numerous and show the marked effects of a change in the cell surface. In experiments here reported 0.01 to 0.05 mg. of lead acting upon 5 billion red cells caused such marked variations from normal as:

1. Partial loss of the normal stickiness of red corpuscles, which is demonstrated by their falling from a clean glass surface.
2. Loss of the agglutination reaction which normally follows mixture with serum of a different isoagglutinating group.
3. Decrease in volume even in isotonic solutions.
4. Loss of normal elasticity and, therefore, reduced changes in volume upon exposure to marked variations in osmotic tension.
5. Increase in resistance to large changes in external osmotic pressure because of this inelasticity, and therefore decreased hemolysis in hypotonic salt solution (Part 1).
6. Increase in the speed of disintegration in spite of this increased resistance to external osmotic pressure. "Leaded" cells break up more readily upon standing than do normal cells, and are easily fractured by rotation or shaking (Part 1).

All these phenomena seem to be associated largely with surface changes in the corpuscles. Evidence is cited that there is no chemical reaction between lead and hemoglobin. The gas exchange is identical in normal and "leaded" cells. The function of the interior of the red cells, therefore, appears to be unaffected by lead.

The effects of lead upon red blood cells are thus manifested by shrinkage, inability to expand, increased brittleness, and loss of the normal consistency which makes their surface sticky. After exposure to lead, red blood corpuscles are more like hard inelastic brittle rubber balls, than like the soft, elastic, resilient cells characteristic of normal blood.

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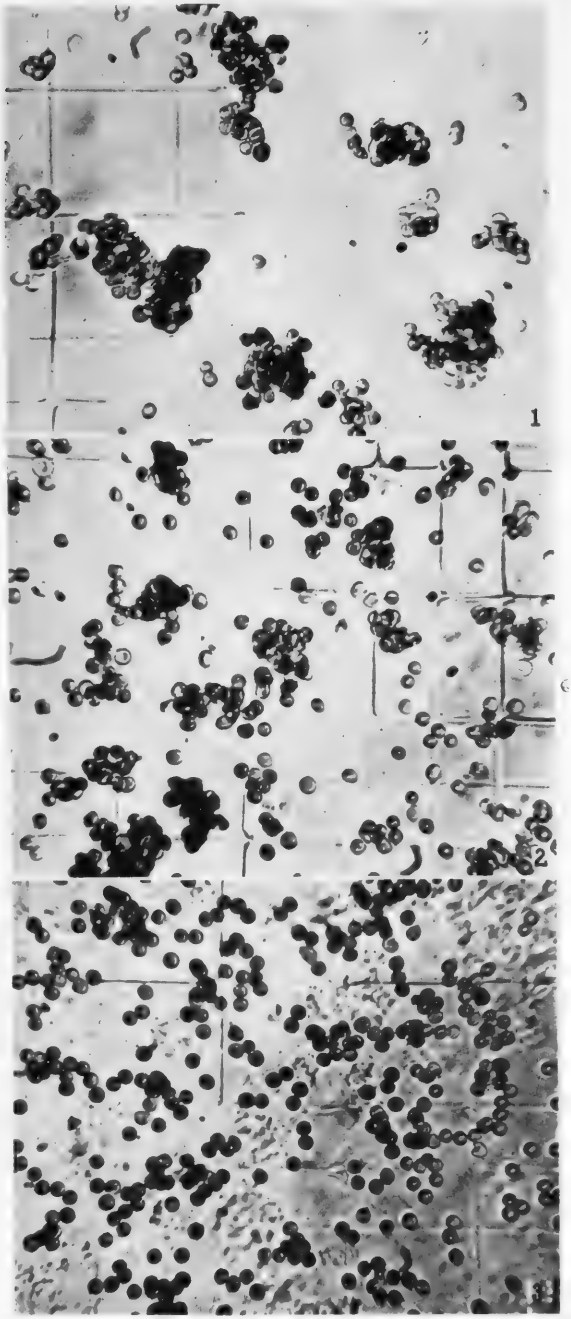
EXPLANATION OF PLATE 5.

Effect of lead on the agglutination of Group II cells by Group IV serum.

FIG. 1. Normal cells exposed only to Ringer solution.

FIG. 2. Cells exposed for 1 hour to 0.015 mg. of Pb per cc. of cells.

FIG. 3. Cells exposed for 1 hour to 0.05 mg. of Pb per cc. of cells.



(Aub, Reznikoff, and Smith: Lead studies. III.)

LEAD STUDIES.

III. The Effects of Lead on Red Blood Cells.

Part 3. A Chemical Explanation of the Reaction of Lead with Red Blood Cells.

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(Received for publication, March 27, 1924.)

The first two papers of this series (1, 2) have dealt with the effect of lead upon red blood cells and have suggested an interaction of the lead with the cell surface as the mechanism underlying the phenomena observed. All the changes probably have their origin in a chemical reaction and it is the purpose of this paper to report experiments designed to study this reaction.

An experiment described in Part 1 suggested a method of investigating this problem. It was found that lead, mixed with small amounts of blood serum, could no longer cause the usual change of resistance of red blood cells to hypotonic saline solution. In other words, some substance in the serum reacted with the lead to prevent further combination with red blood cells subsequently added. In order to ascertain which constituents were involved in this reaction, it was essential to determine the ability of each to combine with and to neutralize lead.

As far as is known at present, red blood cells are composed of inorganic salts, lecithin, cholesterol, albumin, euglobulin, pseudoglobulin, and hemoglobin (3). Each of these substances, in pure form and in amounts equal to its concentration in blood, was mixed with lead. After allowing time for a reaction to occur, red blood cells were added to this mixture and the usual hypotonic saline tests were carried out. If a normal curve was obtained, the sub-

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stance obviously had combined with the lead in the preliminary treatment, and thus prevented the latter from acting on the red cells. On the other hand, if the curve was that characteristic of "leaded" cells, the substance in question had had no effect on the lead. This test, therefore, provides a biological indicator of a chemical reaction involving amounts of lead as small as 0.01 mg. per cc. of red blood cells (1). In this work it was necessary, of course, to simulate as closely as experimental conditions permitted, conditions occurring normally in whole blood. The solutions of blood constituents were prepared with this in view, especially with respect to hydrogen ion concentration and osmotic pressure.

The Effect of the Inorganic Salts of Blood on Lead.

The first substances to be tested were the inorganic constituents of the red blood cells. Of these, the sulfates, carbonates, and phosphates are the most likely to unite with lead. Landolt, Börnstein and Roth (4) quote Böttger's solubility findings as follows:

Lead sulfate	42.5	mg. per liter.
Lead carbonate	1.43	" " "
Lead phosphate	0.13	" " "

From these figures it can be seen that the sulfate is relatively soluble and would not unite with lead in the presence of the more insoluble carbonate and phosphate. On the basis of solubility, moreover, small amounts of lead added to blood would be converted completely to phosphate, provided the reaction between lead and red blood cells were chemical in nature.

Carbonates.—In determining the effect of carbonates on lead, equal parts of double Ringer[1] and sodium carbonate solutions were mixed so that the final concentration was $\frac{M}{40}$ CO_2 which is approximately that of normal serum (5). The pH of this mixture was 6.9. Lead, exposed to this mixture for $\frac{1}{2}$ hour in amounts equivalent, respectively, to 0.02 and 0.2 cc. of serum, was added to washed red blood cells (0.01 mg. of lead per cc. of cells). After exposure for 1 hour, hypotonic saline tests were performed.

[1] By double Ringer solution is meant one with twice the concentration of salts, so that, when diluted with an equal volume of water, it is isotonic with blood.

In a previous paper (1) it was shown that 0.02 cc. of normal serum almost completely neutralizes the effect of 0.01 mg. of lead upon 1 cc. of corpuscles. In the present experiments an amount of carbonate, equivalent to that in 0.2 cc. of serum, only partially neutralized the lead, while that present in 0.02 cc. of serum had almost no effect (Table I). Therefore, union of lead with the carbonates of the corpuscles probably does not explain the "leading" of red blood cells.

Phosphates.—The presence of inorganic phosphates in red blood cells has been questioned recently (6), but it is generally accepted that inorganic phosphate is present in normal whole blood, in a

TABLE I.

Neutralization of the Effect of Lead on Red Blood Cells by Constituents of Blood.

Substance tested.	No. of cc. of solution, equivalent to blood in content of substance tested, necessary to show minimal neutralization of 0.01 mg. of Pb per cc. of red blood cells.	No. of cc. of solution, equivalent to blood in content of substance tested, necessary to show maximum neutralization of 0.01 mg. of Pb per cc. of red blood cells.
Carbonates.	0.02	0.2
Phosphates.	0.004	0.02
Sodium glycerophosphate.	No neutralization.	No neutralization.
Lecithin.	" "	" "
Cholesterol.	" "	" "
Albumin.	" "	" "
Euglobulin.	" "	" "
Pseudoglobulin.	" "	" "
Hemoglobin	" "	" "

concentration of approximately 3.0 mg. of phosphorus per 100 cc. of blood, and appears to be equally distributed between corpuscles and plasma (7).

To obtain a solution with an inorganic phosphorus content equal to that of blood, 0.0232 gm. of NaH_2PO_4 was dissolved in 100 cc. of water and diluted with an equal volume of double Ringer solution. The pH of this solution was then brought to 6.5 with $\frac{N}{10}$ sodium hydroxide. Table I shows that after 0.01 mg. of lead is treated with a quantity of this mixture equivalent to 0.02 cc. of serum, it has no effect on red blood cells added subsequently (1 cc. of cells per 0.01 mg. of Pb). This indicates that the lead was rendered

completely inactive by the preliminary treatment with inorganic phosphate. In these concentrations, therefore, the effect of lead on red blood cells can be entirely explained by the lead-phosphate reaction.

In vivo a preliminary reaction between lead and inorganic phosphate in the absence of red blood cells does not occur (1). Therefore the next point to be considered was the neutralizing effect of phosphate when added to corpuscles or whole blood before exposure to lead. Preliminary tests showed that much more phosphate is required in these cases to affect the action of lead. This, of course, is to be expected because the lead reacts simultaneoulsy with red blood cells and inorganic phosphate—conditions similar to those *in vivo* (see Part 1).

In one series of experiments varying amounts of phosphate were added to washed red blood cells, and after standing for ½ hour the mixtures were treated with 0.01 mg. of lead per cc. of corpuscles. Neutralization of the lead was first observed with an amount of phosphate equal to that in 0.02 cc. of serum, and was found to be almost complete with a quantity of phosphate equal to 0.12 cc. of serum (Table II). It is very significant that concentrations of phosphate greater than that contained in 0.12 cc. serum have no additional neutralizing effect. This shows that some red blood cells are affected by lead no matter what the neutralizing value of the serum may be, provided corpuscles, lead, and serum are all present at the same time. Such a condition, of course, occurs *in vivo* and accounts for the inability of serum to overcome completely the action of lead on the cells.

A similar experiment was carried out with defibrinated whole blood. Varying quantities of phosphate were added to whole blood and after ½ hour the mixture was treated with 0.04 mg. of lead per cc. of blood. It has been shown (1) that 0.04 mg. of lead has nearly the same effect on 1 cc. of whole blood as 0.01 mg. of lead on 1 cc. of washed corpuscles. Phosphate, when added to whole blood in a quantity equivalent to that found in 0.48 cc. of serum, failed to neutralize the lead completely (Table II). Therefore, a mixture of phosphate and whole blood can neutralize less lead than a mixture of the same amount of phosphate and washed

cells from an equal quantity of blood. This fact suggests the possibility that phosphate in Ringer solution is more available to react with lead than is phosphate added to serum. Here again, the failure of excess phosphate to neutralize completely the lead, which acts simultaneously on the corpuscles, illustrates the condition *in vivo*.

There is further evidence that lead reacts with inorganic phosphate. About 0.02 cc. of normal serum can unite with 0.01 mg. of lead to neutralize completely the effect of the lead on 1 cc. of red blood

TABLE II.

Relation of Inorganic Phosphate in Blood to Neutralization of the Effect of Lead on Washed Red Blood Cells and on Whole Blood.

Experiment.	No. of cc. of phosphate solution, equivalent to serum in inorganic phosphorus content, necessary to show minimal neutralization of effect of 0.01 mg. of Pb per cc. of red blood cells.	No. of cc. of phosphate solution, equivalent to serum in inorganic phosphorus content, necessary to show maximum neutralization of effect of 0.01 mg. of Pb per cc. of red blood cells.
1. Phosphate added to lead, before adding red blood cells.	0.002	0.02 (complete neutralization).
2. Phosphate added to red blood cells, before adding lead.	0.02	0.12 (neutralization incomplete).
3. Phosphate added to whole blood before adding lead.	0.04	0.15–0.48 (neutralization less complete than in (2)).

0.01 mg. of lead per cc. of red blood cells and 0.04 mg. of lead per cc. of whole blood are equivalent in effect.

cells (1). Serum with an abnormally high inorganic phosphate content should have a greater neutralizing value. Among several specimens tested, a few were obtained which illustrate this point. In these experiments the serum was diluted with Ringer solution so that small amounts could be added conveniently. The diluted serum was mixed with lead and, after ½ hour, normal red blood cells were added to determine the degree to which the serum neutralized the lead. Three cases are reported here.

The first (J. B. C.) was one of lead poisoning complicated by rather severe nephritis. The inorganic phosphate content was increased to 6.7 mg. of phos-

phorus per 100 cc. of whole blood. His serum neutralized lead between two and three times as effectively as normal serum (Table III).

The second specimen was taken from a patient (O. M. B.) suffering from uremia. The whole blood contained 15 mg. of inorganic phosphorus, 165 mg. of non-protein nitrogen, and 8.01 mg. of creatinine per 100 cc. This serum was four times as effective as normal in its neutralization of lead (Table III).

A third case (S. S.) was one of lead poisoning uncomplicated by nephritis. The blood of this patient contained 4.4 mg. of inorganic phosphorus and 35.7 mg. of non-protein nitrogen per 100 cc. The result shows that 0.012 cc. of this plasma was almost as effective as 0.02 cc. of normal serum. Although in this case the phosphate content was only slightly higher than normal, the neutralizing effect is increased (Table III).

TABLE III.

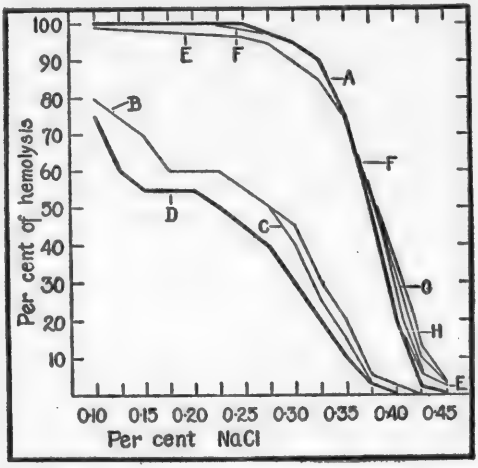
Relation of Inorganic Phosphorus Content of Blood to Amount of Serum Necessary to Neutralize the Effect of 0.01 Mg. of Lead on 1 Cc. of Red Blood Cells.

Serum.	Mg. of inorganic phosphorus per 100 cc. of whole blood.	No. of cc. of serum necessary to obtain minimal neutralization of 0.01 mg. of lead per cc. of red blood cells.	No. of cc. of serum necessary to obtain practically complete neutralization of 0.01 mg. of lead per cc. of red blood cells.
1. L. F. (control).	3.0	0.002	0.02
2. J. B. C.	6.7	0.0006	0.012
3. O. M. B.	15.0	0.0004	0.005
4. S. S.	4.4	0.001	0.012

Other observations point to the importance of phosphate in the union of lead and corpuscles. Experiments were performed in which washed red blood cells were permitted to stand at 30° C. for varying intervals. Without being washed free from the Ringer solution in which they stood, the corpuscles were then treated with lead (0.01 mg. per cc. of cells). Text-fig. 1 illustrates one of seven such experiments and shows that, while the action of lead is at first increased, after 2 hours, small concentrations of lead are no longer effective. The same result was obtained in three experiments performed at room temperature, but it was necessary to allow the cells to stand for longer periods before adding lead in order to prevent the usual effect of the latter.

This change might be due to diffusion of substances from the red blood cells into the Ringer solution. To determine this, washed corpuscles were centrifuged after standing for various lengths of

time and the supernatant fluid was tested for phosphate, carbonate, and protein. The Ringer solution used for washing contained only sodium, potassium, and calcium chlorides; therefore, any other



TEXT-FIG. 1. The effect of lead on red blood cells which have stood at 30°C. for varying intervals before the addition of lead.

A, red blood cells.

B, red blood cells at 30°C. in Ringer solution for 15 min.; then, 0.01 mg. of Pb per cc.

C, red blood cells at 30°C. in Ringer solution for 30 min.; then, 0.01 mg. of Pb per cc.

D, red blood cells at 30°C. in Ringer solution for 1 hr.; then, 0.01 mg. of Pb per cc.

E, red blood cells at 30°C. in Ringer solution for 2 hrs.; then, 0.01 mg. of Pb per cc.

F, red blood cells at 30°C. in Ringer solution for 3 hrs.; then, 0.01 mg. of Pb per cc.

G, red blood cells at 30°C. in Ringer solution for 4 hrs.; then, 0.01 mg. of Pb per cc.

H, red blood cells at 30°C. in Ringer solution for 5 hrs.; then, 0.01 mg. of Pb per cc.

substances present after washing must have diffused from the corpuscles, as there was no evidence of hemolysis. To test for phosphate, molybdic acid was used; for carbonates, 1:1 hydrochloric acid;

and for proteins, both the heat and acetic acid test and Millon's reagent. Table IV shows the results. The failure of lead to act on red blood cells which have stood in Ringer solution for several hours is probably connected with the diffusion of some substance from the cell into the surrounding medium. It is especially to be noted that the phosphate is one of these diffusing substances.

The question arises, as to whether greater concentrations of lead can affect cells from which diffusion has taken place. To test this, washed corpuscles were permitted to stand in Ringer solution at 30°C. for 6 hours. After centrifuging, the supernatant fluid was discarded and the suspension of cells was brought back to its original volume with fresh Ringer solution. Several samples of these erythrocytes were treated with 0.01, 0.02, 0.04, and 0.2

TABLE IV.

Diffusion from Red Blood Cells Standing in Ringer Solution.

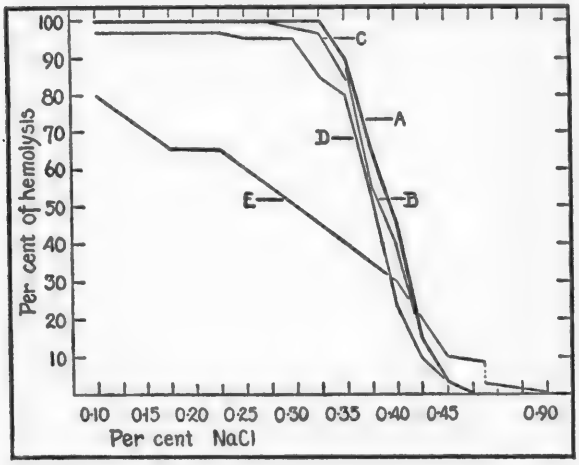
Solution tested.	Phosphates.	Carbonates.	Proteins.	
			Heat and acetic acid.	Millon's reagent.
Ringer solution.	Negative.	Negative.	Negative.	Negative.
Supernatant fluid after 3 hrs.	++	+	"	+
" " " 6 "	+++	++	Very slight trace.	++

mg. of lead per cc. of red blood cells, respectively. The results obtained are shown graphically in Text-fig. 2 and show that (*a*) 0.01 mg. of lead has practically no effect, (*b*) 0.02 mg. begins to "lead" the cells very slightly, (*c*) while 0.04 mg. has a distinct, and (*d*) 0.2 mg., a very marked action.

This failure of small quantities of lead to affect corpuscles which have stood in Ringer solution might be due to neutralization of lead by phosphate which has diffused from the cells. To investigate this point experiments were performed in which red blood cells stood in Ringer solution for varying lengths of time. Part of these were then "leaded" in the original Ringer and part were washed twice with fresh Ringer. In Text-fig. 3 are shown the results of one of three experiments, which illustrate quite conclusively that

when the cells are washed free from the original Ringer they can be affected again by small quantities of lead.

All these experiments point to the importance of inorganic phosphate in the interaction of lead and red blood cells. They show that inorganic phosphate, in the same concentration as normally occurs in serum, combines with as much lead as the whole serum. This



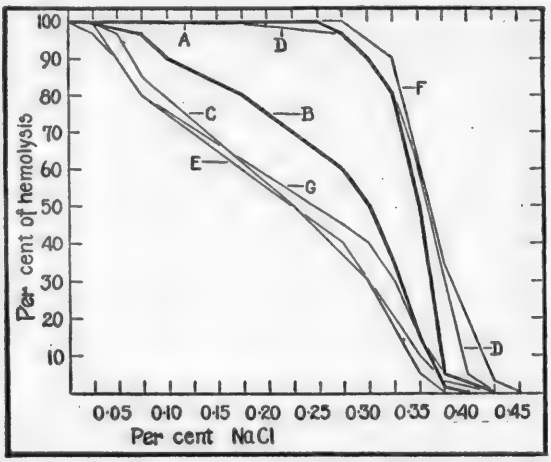
TEXT-FIG. 2. The effect of increasing concentrations of lead on red blood cells which have stood in Ringer solution for 6 hours.

A, control of red blood cells in Ringer solution for 6 hrs.
B, 2.5 cc. of red blood cells and 0.01 mg. of Pb per cc. of red blood cells.
C, 2.5 " " " " " " 0.02 " " " " " " " " " "
D, 2.5 " " " " " " 0.04 " " " " " " " " " "
E, 2.5 " " " " " " 0.2 " " " " " " " " " "

reaction between lead and phosphate is also suggested by the fact that Ringer solution, into which phosphate has diffused from red blood cells, can neutralize lead. Furthermore, serum with a high inorganic phosphate content, and either red blood cells or whole blood to which inorganic phosphate has been added, can react with much more lead than does normal serum or blood.

The Effect of the Blood Lipoids on Lead.

Lecithin.—The organically bound phosphorus compounds of red blood cells were next investigated. Lecithin is the principal of these. Preliminary tests were performed with sodium glycerophosphate. Accepting the formula for lecithin suggested by Levene



TEXT-FIG. 3. The effect of washing red blood cells which have stood in Ringer solution for varying intervals before the addition of lead.

A, red blood cells.

B, red blood cells and lead (0.01 mg. of Pb per cc.). Red blood cells at 31.5°C. for 1 hr. before addition of lead.

C, red blood cells at 31.5°C. for 1 hr.; twice washed before addition of lead.

D, red blood cells at 31.5°C. for 2 hrs. before addition of lead.

E, red blood cells at 31.5°C. for 2 hrs.; twice washed before addition of lead.

F, red blood cells at 31.5°C. for 4 hrs. before addition of lead.

G, red blood cells at 31.5°C. for 4 hrs.; twice washed before addition of lead.

(8), and Abderhalden's figure (9) for the lecithin content of serum—1.675 gm. per liter—a solution was prepared equivalent in phosphorus content to that of the blood by dissolving 0.1048 gm. of sodium glycerophosphate in 100 cc. of Ringer solution. The pH

was brought to 6.7. After treating 0.01 and 0.12 mg. of lead with 0.4 and 2 cc. of the glycerophosphate solution, respectively, for ½ hour, 1 cc. of washed red blood cells per 0.01 and 0.12 mg. of lead was added. In the concentrations used, sodium glycerophosphate had no effect on the subsequent action of lead on the red blood cells (Table I).

Blum (10) has suggested the possibility that a combination of lead with lecithin may be responsible for the symptoms of plumbism. Bloor (11) gives as the concentration of lecithin and cholesterol in human blood 0.30 and 0.25 gm. per 100 cc., respectively. In these experiments an emulsion of lecithin,[2] equivalent to 0.25 gm. per 100 cc., was made in Ringer solution and gave no positive test for inorganic phosphate. 0.4 and 2 cc. of this lecithin preparation (equivalent to 0.03 and 0.17 cc. of blood) were treated with 0.01 and 0.04 mg. of lead, respectively, for ½ hour. Then 1 cc. of red blood cells per 0.01 and 0.04 mg. of lead was added and the results showed that lecithin alone modified the permeability of the cells, but did not alter the usual effect of lead (Table I). It is interesting to note that Thudichum (12) could precipitate all the lipoids of the brain except lecithin with basic lead acetate.

Cholesterol.—Although cholesterol is insoluble in Ringer solution, it can be dissolved in an emulsion of lecithin (3). An excess of cholesterol (Eimer and Amend's C.P.) was added to the lecithin emulsion described above. After filtration, 1 cc. of the lecithin-cholesterol emulsion was found by Bloor's method (11) to contain 0.5 mg. of cholesterol—about the amount present in 0.25 cc. of blood. In the experiment 0.2 and 0.04 cc. of the emulsion were used. These quantities were equivalent in cholesterol content to 0.05 and 0.01 cc. of blood, respectively. These mixtures stood with 0.01 mg. of lead for ½ hour and then 1 cc. of corpuscles per 0.01 mg. of lead were added. The result showed that lead retained its original effect (Table I). Knowing the action of lecithin alone, it seems reasonable to conclude from these observations that cholesterol cannot combine with lead.

[2] Obtained from the Abbott Laboratories, Chicago, and assayed 93 per cent pure.

As a check, lecithin was dissolved in alcohol and an aqueous solution of lead chloride (0.81 mg. of lead per cc.) was added until a precipitate formed. Filtration removed the great bulk of the precipitate, but the filtrate was somewhat turbid. The precipitate was washed repeatedly with acidulated water until the washings were free from lead when tested with hydrogen sulfide. The residue and filter paper were ashed, dissolved in hydrochloric acid and tested for lead. The same procedure was carried out with cholesterol, using acetone as a solvent. In this case the filtrate was clear. With neither lecithin nor cholesterol was lead found in the residue. This indicates that the lipoids of blood did not form an insoluble compound with lead.

The Effect of the Blood Proteins on Lead.

Euglobulin.—The first protein to be tested was serum euglobulin.[3] Normal whole blood contains 50 gm. of globulin per liter, of which 41 per cent or 20.5 gm. is euglobulin (13). According to Hardy (14) this protein contains 0.07 to 0.08 per cent phosphorus.

In these experiments the preparation used was made by Dr. E. J. Cohn (15) from horse serum, contained no inorganic phosphates, a very faint trace of chlorides, and had a pH of 6.49. The nitrogen content was 0.26 gm., equivalent to 1.6 gm. of protein, per liter. When diluted with an equal volume of double Ringer, 1 cc. of the solution contained the same amount of euglobulin as occurs in 0.04 cc. of blood.

Varying quantities of this protein were treated with 0.01 and 0.12 mg. of lead, and the mixtures tested against 1 cc. of red blood cells. Table I shows that the protein has a slight modifying influence on the behavior of red blood cells in hypotonic saline but as much euglobulin as is present normally in 0.16 cc. of blood has no neutralizing action on lead.

Pseudoglobulin.—The pseudoglobulin used was prepared from horse serum (15). It was salt-free and had a nitrogen content

[3] The serum euglobulin, pseudoglobulin, and hemoglobin were obtained from the Laboratory of Physical Chemistry, Harvard Medical School. We take this opportunity to express our sincere thanks to Dr. E. J. Cohn for these materials and for his many helpful suggestions.

of 1.115 mg. per cc., or 7 gm. per liter. The pH was raised to 6.2 and an equal volume of double Ringer solution added. Since normal blood contains 29.5 gm. of pseudoglobulin per liter (13), this procedure gave us a solution 9.4 cc. of which contained an amount of pseudoglobulin equivalent to that normally found in 1 cc. of blood. Treating 0.01 and 0.04 mg. of lead with the amount of pseudoglobulin present in 1 cc. of blood rendered it no less effective than untreated lead in its action on red blood cells (Table I). Pseudoglobulin has a marked water-binding power and therefore modifies the shape of the hypotonic saline curve. With lead the effect is additive.

Hemoglobin.—The hemoglobin employed was prepared from horse blood according to the method of Ferry (16). It was in aqueous solution (0.126 gm. of hemoglobin per cc.). The pH was 7. This was diluted with an equal volume of double Ringer. Since normal blood contains 16.7 per cent of hemoglobin by weight (9), 2.6 cc. of our hemoglobin-Ringer mixture was equivalent to 1 cc. of blood. Experiments showed that hemoglobin in solutions equivalent to 0.2 and 0.75 cc. of blood fails to neutralize the effect of 0.01 and 0.04 mg. of lead, respectively, per cc. of red blood cells (Table I). Hemoglobin alone, like the other proteins, somewhat alters the hypotonic saline curve, but does not affect the action of lead on red blood cells.

Albumin.—Albumin was prepared from horse serum as follows: The serum was half saturated with $(NH_4)_2SO_4$ to precipitate the globulins, and the mixture filtered twice. To the chilled filtrate $\frac{N}{10}$ H_2SO_4, half saturated with $(NH_4)_2SO_4$, was added until the pH of the solution was 5.2. When this solution was allowed to stand overnight crystalline albumin separated out. The crystals were dissolved in water and were twice reprecipitated with saturated $(NH_4)_2SO_4$ solution, the pH being kept constant to allow complete separation of the albumin. Between precipitations the albumin was dissolved in a small volume of water and filtered. The aqueous solution was dialyzed in Dr. Cohn's laboratory until free from all sulfate. It contained 6.22 mg. of nitrogen or 38.87 mg. of albumin per cc.

Since in blood there are approximately 29.4 gm. of albumin per liter (13), 1 cc. of blood contains the same quantity of albumin as 0.76 cc. of our solution. In these experiments isotonic mixtures were made with Ringer solution so that 1 cc. of the mixture was equivalent to 0.1 and 0.5 cc. of blood. The pH of these two mixtures was 6.6. It was found that albumin, even in quantities as great as occur in 1.5 cc. of serum, had no effect on the action of 0.01 mg. of lead upon 1 cc. of red blood cells (Table I).

This negative result is interesting because a lead albuminate is known to exist. If lead is added to serum (0.5 mg. of lead per cc. of serum) a precipitate of lead albuminate is formed if the solution is between pH 5.8 and 6.0 (17); but if the hydrogen ion concentration varies beyond these limits in either direction the precipitate dissolves. Furthermore in the presence of inorganic phosphates, small amounts of lead would combine not with the albumin, but with the phosphate.

The Explanation of the Effect of Lead.

How can the changes in red blood cells caused by lead be best explained? Apparently they depend upon the union of lead with inorganic phosphate, and are largely surface phenomena (2). But is the mere removal of ionized phosphate by lead sufficient to cause these effects? The diffusion experiments prove this improbable. When red blood cells are permitted to stand in Ringer solution for several hours, considerable phosphate diffuses out; nevertheless, despite this loss in phosphate, there is no variation of the hypotonic saline curve from normal.

The possibility that lead phosphate is precipitated on the surface of the cell and has a modifying influence must be considered. It is very probable that phosphate unites with lead as $Pb_3(PO_4)_2$. This may be precipitated in the membrane, where its very presence may change the colloidal properties. That it forms a complete coating on the outside of the cell, however, is hardly probable, because of the very small amount of lead used. Determination of the possible magnitude of this reaction cannot be made, for all the cells are not equally affected by lead and therefore calculations of the amount of lead available for each cell or of the amount of phosphate involved

per unit of cell surface are not justified. A rough approximation made by Dr. Fairhall should, however, be mentioned. It shows that the area of a red cell is at least of the order of magnitude of 10^{-7}, while that of the lead molecules available for that cell is at most of the order of 10^{-12}. Such a great difference makes the possibility of a superficial coating of the cells even by a single molecular layer highly improbable.

TABLE V.

Agglutination of Normal and "Leaded" Red Blood Cells by Colloidal Iron and Arsenic Solutions.

0.1 cc. of corpuscles per cc. of colloid.

Strength of colloid (diluent, isotonic saccharose).	Colloidal iron.*		Colloidal arsenic.*	
	Normal red blood cells.	"Leaded" red blood cells.	Normal red blood cells.	"Leaded" red blood cells.
Full strength.	+	++	++++	++++
1:2	++	++	++++	+++
1:4	++	++++	++++	++
1:8	+++	++++	++++	++
1:16	++++	+++	++++	+
1:32	++++	+++	++++	+

++++ Very marked agglutination.
+++ Marked agglutination.
++ Moderate "
+ Slight "
0 No "

* The colloids were prepared according to directions from Holmes, H. N., Laboratory manual of colloidal chemistry, New York and London, 1922.

Such phenomena as the disappearance of agglutination and the loss of stickiness of the cells (2) suggest a change in the charge of the cell surface as a possible explanation of the action of lead. Rather marked differences in the agglutination of normal and "leaded" blood cells may be observed when tested with colloidal iron or colloidal arsenic. Table V gives the results of one of five experiments.

In view of these variations with colloidal solutions it seemed desirable to determine directly the charge of normal and "leaded" cells by cataphoresis measurements. This was done with an apparatus described by Dr. E. J. Cohn and his coworkers (18). In

the experiments red blood cells were washed free from plasma, part then being "leaded" and part being used as controls. Both control and "leaded" corpuscles were washed three times with isotonic saccharose solution. Distinct differences in direction or rate of migration could not be detected between normal and "leaded" cells, at pH 6.5 or 5. There certainly was no reversal of charge. It must be remembered that the methods of determination are relatively crude and that there are many unavoidable sources of error inherent in cataphoresis measurements of red blood cells. The microscopic method described by Northrop (19) might show differences between normal and "leaded" cells but no attempt has been made to use the method.

Some work by Fairhall (17) introduced another factor in the probable explanation of the action of lead. He found that on mixing lead chlorides and disodium phosphate, a precipitate of lead phosphate is formed and that there is a marked rise of the hydrogen ion concentration to at least pH 2.5. This increase in acidity is due to the liberation of free HCl in the reaction

$$2\ Na_2HPO_4 + 3\ PbCl_2 \rightarrow Pb_3(PO_4)_2 + 4\ NaCl + 2\ HCl$$

Could this acid be of importance in the effect of lead on red blood cells? Although buffer action would prevent any appreciable change in the pH of the blood, could there be a marked local change at the cell surface? If this were the case, there would be a disturbance of colloidal equilibrium with precipitation of the proteins at their isoelectric points, and even coagulation.

The objection may be raised that normally the buffers of the blood are quite adequate to take care of any acid produced. It must be remembered, however, that we are here dealing with a cell surface capable of local reactions and variations in diffusion. Furthermore, the principal buffer, the phosphate, interacts with lead to form insoluble phosphate and free acid, thus diminishing its buffer action. A series of experiments with collodion sacs illustrates this. Mixing equal volumes of Ringer solution containing $PbCl_2$ (0.1 mg. of Pb per cc.) and serum diluted three times with Ringer solution, produces no macroscopic precipitate. If, however, the diluted serum alone is placed in a collodion sac which is suspended in the lead solution,

a heavy precipitate forms at the membrane. Moreover, if methyl red is added to each solution, both remain alkaline to the indicator but the membrane itself turns red. This demonstrates a local formation of acid. The same result is obtained if phosphate solution is substituted for serum.

These facts would imply that anything which changes the colloidal state of the cell surface affects its properties. The two reactions due to lead, are the precipitation of insoluble lead phosphate and the production of acid. These local reactions may not be the only causes of colloidal changes, but they are very important factors in producing the phenomena described in the two preceding papers.

CONCLUSIONS.

The possible reaction of lead with each of the pure chemical constituents of the red blood cell has been studied. As the indicator of chemical combination the effect of lead on the behavior of red blood cells in hypotonic saline was used.

1. By means of such tests it was found that lecithin, cholesterol, euglobulin, pseudoglobulin, hemoglobin, and albumin do not interfere with the reaction.

2. Sodium bicarbonate can neutralize lead; but ten times as much is required as occurs in an equivalent neutralizing quantity of serum. This carbonate can therefore play only a small rôle in the reaction.

3. Inorganic phosphate in the same concentration as is present normally in serum, neutralizes the same quantity of lead as does the whole serum itself. This can, therefore, completely account for the action of lead on red blood cells.

4. Adding inorganic phosphate to red blood cells or to whole blood greatly decreases the action of lead on the cells but cannot eliminate it completely. This indicates that there is a simultaneous reaction between lead and serum and lead and corpuscles when all are present, and explains the action of lead *in vivo*.

5. Serum with increased inorganic phosphate content, obtained from two patients, neutralized the effect of lead to a high degree. In one case serum with a high normal phosphorus content had a similar though less marked action.

6. Diffusion of inorganic phosphate from red blood cells into the surrounding Ringer solution prevents the action of lead upon the corpuscles. This is due to neutralization of the lead by the diffusate. Washing these same cells free from diffusate again allows lead to react with them.

7. All the findings indicate that the action of lead upon red blood cells can be entirely explained by the union of lead with the inorganic phosphate of the cell.

8. Chemical tests are cited which show that interaction of lead salts and phosphate results in the formation of a very insoluble lead phosphate ($Pb_3(PO_4)_2$) and free acid (Fairhall).

9. When a collodion membrane separates solutions of lead chloride and phosphate or serum, lead phosphate is precipitated in the membrane with a local formation of acid.

10. It is suggested that this acid, which is formed as the result of the union of lead and phosphate, may change the physicochemical state of the colloids of the cell surface.

11. Macroscopic cataphoresis determinations demonstrated no difference between the migration of "leaded" and normal cells.

12. In agglutination experiments with colloidal iron and arsenic a marked difference was observed between "leaded" and normal cells. Thus, further evidence of changes in cell surface is added to that reported in former papers.

GENERAL SUMMARY.

These three papers have developed the following facts, which suffice to explain completely the anemia of lead poisoning.

In vitro, the exposure to a very small amount of lead greatly changes the surface of the red blood cells. Their permeability to water is so altered that they shrink and are incapable of swelling as much as normal cells. With this is associated a marked increase in the resistance to different osmotic surroundings—demonstrated by far less hemolysis than normal in salt solution of very low concentrations. The "leaded" cells, however, are relatively short lived, and hemolyze readily as the result of slight trauma. These observations can also be demonstrated *in vivo* as experiments with rabbits with acute lead poisoning show. In addition to these effects on permeability, lead

alters the physical properties of red blood cells so that they lose their normal stickiness and are no longer agglutinated by the sera of different isoagglutinating groups. All these changes are evidence of an effect on the surface of the cell; the interior of the cell does not undergo disturbances; at least, the physiological properties of the hemoglobin remain normal. The chemical reaction which causes these physical changes in the cell is a precipitation of insoluble lead phosphate and a formation of acid. This causes the "leaded" red blood cell to change from an elastic distensible sac, to one which is contracted, relatively inelastic and brittle. In such condition the cell can poorly withstand the trauma involved in circulation of the blood, and this lack of resistance probably explains the marked destruction of peripheral blood in lead poisoning.

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DIET AND TISSUE GROWTH.

II. The Regeneration of Liver Tissue during Nutrition on Inadequate Diets and Fasting.*

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(Received for publication, April 3, 1924.)

In a previous paper (Moise and Smith (1924)) the postulate was made that nutritive conditions which are ideal for tissue regeneration probably likewise suffice for growth and the experimental results bore out the theory. In the present investigation the aim is to examine the influence of inadequate diets on the rate of tissue regeneration. From our point of view such a diet may be one on which either maintenance of, or increase in body weight is impossible.

The methods available for experimentally inhibiting growth or stunting are numerous. The most obvious is that of underfeeding with an adequate diet. It may be called quantitative stunting, for the animal is prevented from growing because it receives too few calories. Another method that may be called qualitative stunting involves the use of a diet adequate in calories but either lacking entirely, or containing an insufficient amount of, some indispensable constituent such as one of the accessory food factors, essential amino acids, or requisite mineral salts. In the present experiments diets complete in every respect save that they contained a protein which was deficient in one or more of the physiologically necessary amino acids, were used to restrict body growth.

In 1906 Willcock and Hopkins (1906–07) presented data showing that mice declined and died when fed a purified diet in which zein provided all the nitrogen. The decline was delayed but no growth obtained when the amino acid tryptophane was added to the diet. These experiments were repeated by Wheeler (1913) who

* Part of the expenses of this investigation was defrayed from the Russell H. Chittenden Research Fund.

found that, when tryptophane was added to the zein food, her mice maintained their weight for 60 days. It appears that when the lacking tryptophane is added to zein in an otherwise adequate diet, the experimental animal can maintain itself at a more or less uniform weight but cannot grow.

On the other hand, certain proteins can furnish the proper kinds of amino acids for maintenance but not for growth. Gliadin, the prolamine from wheat is one of these. The classic work of Osborne and Mendel (1912) on this protein has indicated the nature of the deficiencies measured by the biological method. They found that if a diet containing gliadin as practically the sole source of protein[1] was fed to rats the animals would remain stationary in weight or grow very slowly if no change was made in the ration. The addition of the amino acid lysine or small amounts of other proteins yielding lysine, resulted in immediate resumption of growth. This information was later applied in experiments (Osborne and Mendel (1914)) with zein with the result that rats fed zein and tryptophane and lysine grew as well as those fed gliadin and lysine and both these groups grew like rats whose diet contained a complete protein, such as lactalbumin, as the source of nitrogen.

A similar, though more severe, inadequacy is involved when gelatin is used as the source of the nitrogen in the food. Early workers were aware that this protein would not suffice for nutritive well-being (von Voit (1881)). Experimental animals maintained in a positive nitrogen balance immediately suffer a negative nitrogen balance when gelatin is included in the diet as the sole protein (von Voit (1881), Örum (1879), Abderhalden and Hirsch (1912)). A glance at the list of amino acids yielded by gelatin on hydrolysis, shows that tryptophane, cystine, and tyrosine are lacking. On the other hand, gelatin which yields lysine can supplement gliadin and so cannot be considered worthless in nutrition, a point early emphasized by von Voit (1881). Kauffmann (1905) showed that gelatin nitrogen could replace as much as one-fifth of the nitrogen of casein in a food mixture without altering the nitrogenous efficacy of the diet. Employing pigs at an endogenous plane of protein metabolism, McCollum (1911–12) demonstrated that more than one-half of the nitrogen of the gelatin was available to the animal, while Murlin (1907) in experiments on man, substituted two-thirds of the nitrogen of meat with gelatin nitrogen without observing a negative nitrogen balance.

The effect of an inadequate diet on the rate of growth of transplanted tumors has been studied by Benedict and Rahe (1917). The ration employed by them was deficient only in vitamin B. Merely enough of a source of this food factor was given to maintain the animal. Under these conditions it appears that the tumor grew though the host could not. Drummond (1917) working with rats used diets containing low percentages of adequate protein as well as diets containing deficient proteins in an effort to influence the growth of tumors in these animals but found that any inadequacy which stopped the growth of the neoplasm prevented even maintenance of the host. He also found that vitamin deficiency

[1] Their diets contained protein-free milk.

inhibits the tumor growth. The effect of gelatin feeding on the regeneration of liver cells after chloroform poisoning in dogs was studied by Davis and Whipple (1919), but no conclusions were drawn from the small number of observations made.

Method.

The plan of the present investigation was to study the rate of regeneration of hepatic cells in the white rat after chloroform poisoning (1) on an otherwise adequate diet containing gliadin as the protein, (2) on a diet containing gelatin as the protein, and (3) in fasting. The liver was used merely because in this organ a uniform type and degree of tissue injury can be produced. The animals were killed at 24 hour intervals up to 168 hours after the administration of the chloroform and the livers sectioned and studied histologically. The technique of the care of the animals was the same as that outlined in the previous paper (Moise and Smith (1924)).

The gliadin food was made up as follows:

	per cent
Gliadin	18
Starch	51
Lard	18
Butter fat	9
Salts*	4

The gelatin food had the following composition:

	per cent
Gelatin (powdered)	18
Starch	51
Lard	18
Butter fat	9
Salts*	4

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

Vitamin B was provided by 100 mg. of dried yeast fed apart from the synthetic food, while the butter therein contained the fat-soluble factor. The energy value was about 5.3 Calories per gm. The diets fed were thus adequate save for the quality of the protein.

The experiments with the gliadin diet were conducted on rats which had grown to at least 70 gm. on a comparable adequate casein diet before being changed to the experimental food. Immediately upon making the change growth stopped and the animals remained at a practically constant weight level. The supplementary

nitrogen of the yeast (7 mg. per day) had no more effect in making good the gliadin deficiencies in these experiments than had the residual nitrogen of the protein-free milk in the experience of Osborne and Mendel. Chloroform was administered only after growth had been inhibited for at least 5 days.

On the gelatin ration the animals did not maintain themselves but lost weight steadily though not so fast as the rats without food. The chloroform was given when the decline on this diet had progressed for at least 2 days.

The loss of weight was more rapid when no food was given and the chloroform was administered usually 2 days after food had been removed from the cages. In order to allow for the rapid decline in weight during the 2 day foreperiod and for the 7 day afterperiod, rats weighing about 200 gm. were employed—animals considerably larger than were used in the other phases of this investigation.

The chloroform used was dissolved in sterile mineral oil and injected subcutaneously. The dosage was so adjusted that the total volume of fluid injected was 1 cc. The chloroform was redistilled, and the fraction 61°–62°C., was used within an hour to obviate the toxic effects of decomposition products due, presumably, to oxidation.

The maximum non-lethal dose of chloroform for the animals on the gliadin food was 1.5 cc. per kilo of body weight. This is the same quantity used for animals on the standard balanced ration and on the high carbohydrate diet.[2] For the rats on gelatin diet the amount of chloroform giving maximum liver injury and minimum number of deaths was 1 cc. per kilo of body weight while the rats receiving no food withstood 1.2 cc. per kilo. The comparative toxicity in these various inadequate dietary conditions is, therefore, as follows: gelatin > fasting > gliadin.

A detailed description of the injury and progressive repair of the liver cells after chloroform poisoning in animals which were fed various adequate diets both of normal and of unusual compositions was presented in a previous paper (Moise and Smith (1924)). In the present account frequent reference and comparison with the findings in animals on the balanced diet is made. A brief description of these results is therefore given.

Repair of the Liver. Standard Diet.—There is an extensive hyaline necrosis involving from two-thirds to four-fifths of the central portion of each liver lobule. After 48 hours there occurs a simultaneous infiltration of leucocytes, a clearing away of the cellular detritus and an active repair of the injury. The process of repair is most active during the 72 hour period, when there are very large numbers of mitotic figures in the uninjured cells at the periphery of the lobule.

[2] See Moise, T. S., and Smith, A. H., *J. Exp. Med.*, 1924, xl, 13.

In the 96 hour sections only slight evidences of the injury or of an active reparative process are seen. After 120 to 148 hours the regeneration is complete and the liver is histologically normal.

Gliadin Diet.—The extent of the injury is about equal to that described on the standard diet. The rate of procedure of the repair is almost identical with that observed on the standard balanced diet. The percentage of the injured area that has been repaired at 48 and 72 hours is practically the same; although the number of mitotic figures is slightly less than in the corresponding periods on standard diet. In the 96, 120, and 144 hour periods only the following slight differences are noted. There is a slightly greater amount of fat in the region about the central vessels and in one instance (gliadin Rat 387, 144 hour period) there are small groups of connective tissue and multinucleated giant cells. These have been described by Pearce (1906) whose interpretation is that these giant cells result from direct division of the slightly injured hepatic cells at the margin of the necrotic area. These differences are so insignificant and so rarely present that they were not mentioned in a preliminary report of this phase of the work (Smith and Moise (1922–23)).

Gelatin Diet.—The necrosis involves from two-thirds to three-fourths of the liver lobule and is no more marked than that observed on the adequate diets (Moise and Smith (1924)). The process of repair is, however, definitely delayed. In the 48 hour period there are very few mitotic figures. There are quite marked variations in the size and the chromatin content of the nuclei of the liver cells. In the 72 hour period, active evidence of regeneration of the liver cells is still absent. In one section there is definite evidence of endothelial and connective tissue proliferation. A few mitotic figures are seen in the proliferating endothelial cells. In the 96 hour period, small necrotic areas persist and there is slight evidence of endothelial proliferation. In the 120 and 144 hour sections there are a few scars and small fatty accumulations in the central portion of some lobules but the greater part of the liver appears normal.

Fasting.—The necrosis involves about two-thirds of the liver lobule. In the 48 and 72 hour periods the reparative process is somewhat less active but is otherwise identical with that described for the standard and gliadin diets. In the 96 hour period there are

small unregenerated areas and occasional scars seen. Some sections still show mitotic figures in the liver cells. In the 120 hour section there is some scarring and slight fatty accumulation in the central zones of the lobules. The 144 hour sections appear normal.

DISCUSSION.

From the data given above it appears that chloroform is no more toxic to animals fed gliadin as the sole protein in an otherwise balanced ration than to those given a complete protein. Actual fasting exerts a definite effect on the resistance of the animal to chloroform, the maximum non-lethal dose being smaller than with the rats on gliadin food. The animals declining on gelatin food were still more sensitive to chloroform although losing weight somewhat less rapidly than those without any food. The more pronounced amino acid deficiency, probable decreased calorie intake due to physical character of the gelatin diet, together with the specific dynamic action of the amino acids in the gelatin may account for the increased toxicity of the poison in this group over that in the fasting group. It is worthy of notice, too, that chloroform is less toxic to animals without any food than to animals fed diets high in fat (Moise and Smith (1924)).

As previously stated the regenerative process is most active on the standard balanced diet. On this ration there is a rapid repair of the injury with a complete return of the liver to the normal anatomical picture without evidence of scarring after 120 to 144 hours.

On the gliadin diet the regeneration is identical with that observed on standard diet with the following insignificant variations. The numbers of mitotic figures are somewhat small on gliadin diet. There is slightly more fat in the central zones of the liver lobules in the 120 and 144 hour sections. In one instance an atypical proliferation of the liver cells resulted in small groups of multinucleated giant hepatic cells (Pearce (1906)). The repair is complete after 120 to 144 hours.

On the gelatin diet the necrosis is no more extensive than was observed on the preceding diet but the process of repair is less advanced at a given period. There is some evidence of cicatrization as was described on high fat and high carbohydrate diets in a previous

paper (Moise and Smith (1924)). These scars are seen in the 72, 96, 120, and 144 hour periods. Except for these small areas of fibrosis and slight fat accumulations in the central zones the repair is complete in the 120 and 144 hour sections.

Under conditions of fasting the necrosis is no more extensive than on the standard, gliadin, and gelatin diets. The process of repair is identical with that described on gelatin diet.

In comparing these inadequate diets, gliadin and gelatin, and starvation, with standard diet, it is seen that the rate of procedure of the repair is practically the same on the standard and gliadin diets. Under conditions of fasting and on a gelatin diet the reparative process is slightly but about equally delayed, while on these latter diets small scars are observed.

In a previous communication (Moise and Smith (1924)) it was suggested that these scars possibly resulted from the delay in repair as they are most conspicuous on a high fat diet, on which the toxicity of the chloroform is greatest and the rate of repair is delayed to a greater extent than on any other diet studied.

In comparing the results of the reparative process in inadequate nutritive conditions, (*i.e.* on gliadin food, gelatin food, and in fasting) with the repair on adequate diets (standard food, high carbohydrate food, high protein food, and high fat food) the most rapid healing is observed on the standard balanced diet and the greatest delay in the repair is observed on the high fat diet. The fasting and the gelatin-fed animals show a somewhat less marked delay than is seen in those on the high fat diet. The animals on carbohydrate diet and on protein diet show a more rapid repair than those fasting or on gelatin food, while the changes in the gliadin-fed animals are almost identical with those on standard diet.

The results of the present investigation indicate that the gliadin diet, one having certain obvious deficiencies, while not sufficing for body growth, does permit repair of damaged liver tissue at the same rate as that in an animal provided with an adequate food. We are dealing with the possibility of growth of some of the component parts while the body as a whole cannot grow. Osborne and Mendel (1912) have cited examples of female rats stunted with gliadin, which became pregnant and gave birth to litters. While these experiments

raise practically the same questions as does the present investigation, they can scarcely be accepted as proof of bodily synthesis of essential amino acids since gliadin does yield a small quantity of lysine on hydrolysis (Osborne (1924)). The protein-free milk may have provided some lysine and the possible contribution of the body tissues is one that has not been adequately evaluated.

In the present series of experiments we find certain tissue—hepatic tissue—not only regenerating on a diet (gliadin) on which body growth is impossible but regenerating as rapidly as the same tissues in animals fed a so called adequate food. It might be argued that the "complete set" of amino acids necessary to rebuild the damaged tissue arises from the breakdown of other tissues such as, for instance, the muscles. We have found that immediately following the chloroform injection the animal appears anesthetized; for 2 or 3 days after the injection the rat is definitely "sick" and eats little, and the curve of body weight drops. However, after the repair is histologically complete and often before that stage is reached, the body weight has regained its original stunted level. These facts seem to show that other body tissue was not breaking down to furnish the amino acids required for hepatic tissue synthesis. Again, Lindsay (1911) and Davis, Hall, and Whipple (1919) have demonstrated that chloroform poisoning is followed by a prompt rise in nitrogen output indicating loss of the hepatic protein nitrogen which, in turn, makes it appear unlikely that all the amino acids necessary for the synthesis of new hepatic tissue come from the necrosed liver cells. One is forced to conclude either that hepatic tissue requires far less lysine than muscle and other body tissues or that correlated growth resulting in an increased body weight is not identical with nor dependent on the same mechanism as individual tissue growth. In the present experiments we are not dealing with an absolute lack of lysine, for this amino acid may be provided (1) in the gliadin to the extent of 0.6 per cent, (2) in the 44 mg. of yeast protein, and (3) possibly by the residue of broken down liver cells. Since we do observe normal liver regeneration under these dietary conditions it appears that there is here a clear demonstration of the quantitative difference between the requirement for the growth of a part and that for the increase of the organism as a whole. It is worthy of note that animals

on the gelatin diet in which the deficiency is more pronounced than in the case of gliadin, are still able to regenerate the liver, though, to be sure, at a slightly less rapid rate. The progressive loss in weight of the rats eating the gelatin food, together with the commonly observed negative nitrogen balance under these conditions indicate that body tissue is being broken down. It is conceivable in this case, that the requisite amino acids for the synthesis of new hepatic cells are provided largely by this endogenous catabolism of proteins.

SUMMARY.

The relative toxicity of chloroform given subcutaneously to rats fed on two diets deficient in respect to their nitrogenous components, the sole sources of protein in which were, respectively, gliadin and gelatin and to rats without food, is as follows: gelatin diet > fasting > gliadin diet.

The rate of regeneration of liver cells after chloroform poisoning in rats fed a diet in which gliadin is the only protein is about as rapid as that in animals fed a diet containing casein as the source of nitrogen. The rate of regeneration on a food containing its nitrogen as gelatin and also during fasting is definitely slower than that in animals fed the gliadin food.

The results of these experiments indicate that the requirement for the essential amino acids for growth of individual organs of the body is less than that for correlated growth of the whole body.

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SOME CULTURAL CHARACTERS OF BACILLUS ABORTUS (BANG) WITH SPECIAL REFERENCE TO CO_2 REQUIREMENTS.

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(Received for publication, April 4, 1924.)

The observations to be described originated in the desirability of knowing whether or not the use of living cultures of *Bacillus abortus* as vaccines may lead to actual cases of disease of the bovine placenta in vaccinated animals or in unvaccinated animals associated with them. This occurrence can be proved by identifying the vaccinal strain isolated from tissues of actual cases of disease. To do this, the vaccinal strain must have acquired characters different from the fresh virus and moreover such as are not lost again in contact with living tissues of the cow.

Strains of *Bacillus abortus* freshly isolated from spontaneous cases differ from those which have been under cultivation for 1 or more years in that the former are sensitive to the atmosphere of the culture tube or environment. The latter are not in so far as they readily multiply in ordinary cotton-plugged tubes on the ordinary agar medium. B. Bang[1] was cognizant of this peculiarity of freshly isolated strains and used serum-gelatin-agar in a deep layer or column. In his hands the bacillus multiplied in a zone 0.5 cm. below the surface. Nowak[2] succeeded in growing *Bacillus abortus* on the surface of solid media by enclosing inoculated tubes in a jar with actively growing cultures of *Bacillus subtilis*. In 1911, M. Fabyan and the writer obtained satisfactory growth by sealing the culture tube hermetically with sealing wax. These several methods lend themselves to several

[1] Bang, B., *Z. Tiermed.*, 1897, i, 241.

[2] Nowak, J., *Ann. Inst. Pasteur*, 1908, xxii, 541.

different interpretations. A choice among them is rendered difficult by the fact that *Bacillus abortus* in its cultural state is an obligatory aerobe without capacity to ferment any sugars.

In order to approach an interpretation of these rather anomalous and apparently contradictory characters, it became necessary to use as a basis for controlling the various experimental departures such methods as made it possible to estimate quantitatively the aerophobic character of the strains used. Growth in sealed tubes was found satisfactory for this purpose and therefore was made the basis of the following study. A number of interesting details were noticed which were of use later in interpreting the results of using gas mixtures. Fortunately a steady succession of over 50 freshly isolated strains was available and what follows bears only on first or second transfers.

The Multiplication of Bacillus abortus in Sealed Tubes as a Function of the Number of Living Bacteria Transferred.—If bits of tissues of bovine fetuses or bits of spleen from inoculated guinea pigs be transferred to agar slants and the tubes sealed, colonies will appear within 7 to 10 days. If transfers of these are made on ordinary meat-infusion-peptone-agar, the subsequent appearance of growth will depend upon the number of bacteria transferred.

To make the operations from strain to strain uniform and quantitative certain arbitrary dilutions were chosen. These were made with a capillary pipette standardized in drops, and a platinum loop, holding about 1/300 cc. The test-tubes were standardized with regard to length and diameter and the agar with regard to batch, quantity, area of slope, and quantity of condensation water. The sealing with sealing wax requires some care both in the making of the seal and subsequent handling of the tubes since slight holes at the start or cracks forming later will affect the growth. Inasmuch as films of growth of the various strains in sealed tubes differed but slightly in density, the entire film was used as a starting point for the dilutions. This film is readily washed down into the condensation water which was about 1 cm. deep and 1/4 cc. in volume. The resulting turbid suspension is free from clumps.

If a loopful of this turbid condensation water is rubbed over an agar slope and the tube sealed a film of growth appears quite regu-

larly within 3 to 4 days. If the tube is not sealed growth usually fails entirely or rarely individual colonies may appear. If the loopful of condensation water is successively diluted two things happen. The appearance of growth is delayed in the lower dilutions and completely suppressed in the higher. Thus in one series of tests growth appeared within 3 days when a loopful of the turbid condensation water was rubbed over the agar slope. The growth with 1/6 loopful appeared in 4 days, with 1/75 in 5 days, with 1/300 in 12 days. All further dilutions had failed when the experiment was closed on the 17th day. Calculations based on plate cultures indicate that the original loopful of condensation water contained between 2 and 3 billion bacteria. Taking the lowest estimate of 2 billion, we note that a 1/300 dilution grew in 12 days, whereas the next, 1/1,800, failed. Into this tube were put slightly more than 1 million, or about 125,000 bacteria to the square centimeter of agar surface. Parallel cultures in unsealed tubes, as stated above, usually fail to develop even when 2 or 3 billion bacteria are transferred. A slight increase may, however, be obtained when the transfer of bacteria is heavy enough to appear as a faint clouding of the agar surface. Then a slight thickening of the film may be observed within 2 days and sometimes irregular local patches of growth.

This phenomenon of delay and suppression has been observed by others in cultures of pneumococci. Gillespie[3] found that a certain number of pneumococci must be added to bouillon to obtain multiplication. Thus one loopful of heart's blood from a mouse was sufficient to start cultures in 5 cc. of bouillon but not in 500 cc. Gillespie calculated that from 1 to 10 million more of pneumococci are needed to start growth in bouillon than on agar.

The phenomenon of delay or lag in the multiplication of pneumococci in fresh culture media was studied in detail by Chesney[4] in 1916. In his communication will be found a statement on the historical development of the concept of lag.

Not infrequently the delayed eruption of a film of colonies of *Bacillus abortus* in sealed tubes may be preceded 1 or more days by the appearance of a few scattering colonies. When the second rich crop appears the first colonies are easily recognized by their greater size. Very rarely a third crop may appear. Occasionally a higher dilu-

[3] Gillespie, L. J., *J. Exp. Med.*, 1913, xviii, 584.

[4] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

tion tube may erupt into view before a lower. In this case an imperfect seal may have influenced the outcome. On the whole however, the order of visibility of the films may be predicted in nine cases out of ten. The multiplication of the freshly isolated *Bacillus abortus* strain is thus a function of the number of living bacilli transferred. There is a threshold value of numbers below which growth fails completely. The multiplication is probably focal depending on cooperation of a certain number of bacteria. Of these a large proportion may die.

The multiplication of *Bacillus abortus* in sealed tubes of bouillon is less in evidence than on agar sloped. Minute doses fail altogether. The special conditions involved have not been studied. It is highly probable, however, that the tendency to continual dilution and dissipation of any cooperative support is largely responsible for the unsatisfactory growth in bouillon.

If we compare with the freshly isolated strains one under cultivation for several years the difference is readily noted. Such a strain will multiply promptly in *unsealed* cultures in dilutions far exceeding those in which growth is still *suppressed* in sealed tubes of fresh strains. In fact it is probable that no living bacteria are suppressed in the saprophytized strains. The change from one state to another may be traced in a series of transfers in which the time for the eruption of the film of colonies is gradually shortened and colonies begin to appear in unsealed tubes. Whether this change is considered a mutation or a more gradual raising or lowering of certain functions remains to be determined.

It had been noted before[5] that the parasitic state is not regained by contact with living tissues of the guinea pigs for 2 or 3 months. On the other hand this contact does not lead to a more saprophytic state. In a number of cases *Bacillus abortus* was isolated both from the fetal tissues directly and from guinea pigs inoculated therewith. The contact of *Bacillus abortus* with guinea pig tissues for 2 to 3 months produced either no change or else a barely recognizable deviation towards the more saprophytic condition. Bacilli readily growing in open tubes injected into the ducts of a cow's udder

[5] Smith, T., and Fabyan, M., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxi, 549.

failed to show any change towards the original condition even after nearly 4½ months stay in the udder and recovery through guinea pigs 2 to 3 months after the injection of the milk into them. We cannot, however, infer from this experiment that a return to the parasitic or animal state does not take place in the placental tissues of the cow. This particular test has not been made. In view of the fact that in several instances the saprophytic stage has been isolated directly from aborted fetuses the inference is warranted that *Bacillus abortus* is not restored to its original animal state by one passage through the fetal membranes of a pregnant cow.

The CO_2 Requirements of Bacillus abortus.

The observations of Bang and the sealed tube suggested that a reduced oxygen tension underlay the peculiar growth characteristic of *Bacillus abortus*, whereas the method of Nowak suggests both reduced O tension and the need for CO_2. This last idea is new as applied to parasitic bacteria. The significance of CO_2 in the activities of nitrifying bacteria had been first brought to light by Winogradsky. The writer has endeavored to trace the origin of the idea that CO_2 may be a necessary factor in the multiplication of pathogenic bacteria. The substitution of the concept of the need of CO_2 in place of that of a reduced oxygen tension has been gradual. The date of its emergence as a definite fact has not been clearly located. Nowak in describing his method of associating cultures of *Bacillus subtilis* regarded the growth as due to reduced oxygen tension. The development of the need of CO_2 has circled at first about the cultivation of the gonococcus and the meningococcus.

In 1916, Wherry and Oliver[6,7] studied the gonococcus and several other organisms by using Nowak's original method of associating the cultures with *B. subtilis*. The better growth was interpreted as due to lowered oxygen tension. In 1918, Wherry and Ervin[8] showed that if culture tubes of the tubercle bacillus be connected with tubes containing barium or sodium hydroxide to absorb CO_2

[6] Wherry, W. B., and Oliver, W. W., *J. Infect. Dis.*, 1916, xix, 288.
[7] Wherry, W. B., and Oliver, W. W., *J. Infect. Dis.*, 1917, xx, 28.
[8] Wherry, W. B., and Ervin, D. M., *J. Infect. Dis.*, 1918, xxii, 194.

the growth was inhibited. Control cultures connected only with tubes of distilled water developed normally.

Cohen and Fleming[9] in isolating meningococci used mixtures of CO_2 and air. Rich growths were obtained with *B. subtilis*, 10 and 20 per cent CO_2. In 30 per cent CO_2 the growth was still good, scant in 50 per cent, and scarce in 75 per cent. This gas is not referred to as a factor however. The authors probably regarded it as simply reducing the oxygen tension. C. W. Chapin[10] in the same year reported a much better growth of primary cultures of the gonococcus in 10 per cent CO_2. His medium was however quite complex.

The significance of a reduced oxygen tension or the presence of CO_2 was challenged by a number of authors. Gates[11] emphasized the importance of moisture secured indirectly by the devices used in cultivating bacteria in gas mixtures. He also showed that the change in reaction due to CO_2 may be a determining factor. In his hands the meningococcus grew well in 15 to 40 per cent O. St. John[12] agrees with Gates in his interpretation of earlier work with CO_2 mixtures. Similarly Kohman[13] infers from a series of experiments with two freshly isolated strains of meningococci in an atmosphere of 10 per cent CO_2 that the favoring influence consists in adjusting the reaction. On the other hand Ruediger[14] obtains successful primary cultures of gonococci by simply closing the tubes with cork stoppers. In 1920, Herrold[15] reports the successful use of both *B. subtilis* and CO_2 mixtures in obtaining plate cultures of the gonococcus. He refers his success to the partial O tension.

Rockwell and McKhann,[16] in 1921, cultivated the gonococcus in various gas mixtures, using the hay bacillus as a kind of control. Although growth was obtained in a pure hydrogen atmosphere, it failed when anaerobic conditions were established with caustic soda and pyrogallic acid. In a further study of a variety of bacteria (tubercle, hay, and anthrax bacilli, gonococcus, and staphylococcus) Rockwell[17] concludes that CO_2 in some way favors certain aerobic and facultative anaerobic species.

In 1922, Erickson and Albert[18] stated that reduced O tension is of no value in cultures of the gonococcus and that moisture is the important factor in the method used. Torrey and Buckell[19] come to the same conclusion.

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[9] Cohen, M. B., and Fleming, J. S., *J. Infect. Dis.*, 1918, xxiii, 337.
[10] Chapin, C. W., *J. Infect. Dis.*, 1918, xxiii, 342.
[11] Gates, F. L., *J. Exp. Med.*, 1919, xxix, 321.
[12] St. John, J. H., *Med. Rec.*, 1919, xcv, 184.
[13] Kohman, E. F., *J. Bact.*, 1919, iv, 571.
[14] Ruediger, E. H., *J. Infect. Dis.*, 1919, xxiv, 376.
[15] Herrold, R. D., *J. Am. Med. Assn.*, 1920, lxxiv, 1716.
[16] Rockwell, G. E., and McKhann, C. F., *J. Infect. Dis.*, 1921, xxviii, 249.
[17] Rockwell, G. E., *J. Infect. Dis.*, 1921, xxviii, 352.
[18] Erickson, M. J., and Albert, H., *J. Infect. Dis.*, 1922, xxx, 268.
[19] Torrey, J. C., and Buckell, G. T., *J. Infect. Dis.*, 1922, xxxi, 125.

In a more elaborate study of various freshly isolated species of bacteria, Rockwell[20] concludes that certain obligatory aerobes may grow without O if CO_2 is present and that some anaerobes need the presence of CO_2 as well as the absence of O.

Struck with the peculiar behavior of the newly discovered bacillus in deep serum-gelatin-agar tubes Bang[1] and his assistant Stribolt carried out a series of tests in 1897 with different oxygen tensions and with CO_2. The results led them to advance the theory of two optima for oxygen, one below and the other much above the atmospheric tension. They regarded CO_2 as of no influence on multiplication. Inasmuch as they used serum and gelatin in the agar and made no statement as to the total age of the cultures employed, their results cannot be interpreted without an exact repetition of their experiments with fresh and old strains.

In 1921, I. F. Huddleson[21] made some experiments with *B. abortus* in an atmosphere containing CO_2. He states that 10 per cent CO_2 is the most favorable concentration and that amounts over 10 per cent are inhibitory. The conception underlying his experiments appears to be that 10 per cent CO_2 brings the pH of the enclosed atmosphere to the point reached by cultures of *B. subtilis* (6.6) and that therefore 10 per cent CO_2 is the proper concentration. C. P. Fitch[22] following Huddleson used 10 per cent CO_2 but states that 10 per cent H is as favorable as CO_2.

The differences of opinion as to the function of CO_2 in stimulating multiplication of certain bacteria may have their origin in at least three possibilities. One is a rapid change of the freshly isolated bacteria in cultures, another is the possible existence of strains already indifferent to CO_2 when first isolated, a third is the favoring action of adherent exudates, blood and the like, in primary cultures and the variable content of these substances in the seed.

Bacillus abortus lent itself very well to a test of the significance of CO_2 in that it multiplies on a relatively simple, easily standardized substrate. As stated above, there was available a succession of fresh cultures in sufficient numbers to control irregularities in the original strain and to avoid working with any but direct transfers from the original culture. Moreover it is easily shown that oxygen is necessary to the growth of *Bacillus abortus*. When this organism has reached a stage in which it multiplies in unsealed tubes the growth is always more vigorous in the unsealed tubes and may be increased in vigor in the sealed tubes, where it soon tends to remain

[20] Rockwell, G. E., *J. Infect. Dis.*, 1923, xxxii, 98.
[21] Huddleson, I. F., *Cornell Vet.*, 1921, xi, 210.
[22] Fitch, C. P., *J. Infect. Dis.*, 1922, xxxi, 233.

stationary, by unsealing the tube. In two tubes, one unsealed, the other sealed, in which multiplication of a partly saprophytized strain has gotten under way, the former always ends up with the heavier crop of bacteria.

The growth in sealed tubes having been standardized both as to quantity of seed and lag, the next step was to determine the effect of an atmosphere without CO_2 under seal. This condition was created by hanging a small test-tube containing about 0.5 cc. of a normal or stronger solution of caustic soda in the upper part of the culture tube just below the plug. The effect of this arrangement on the growth is inhibitory and even more so than the merely unsealed tube would be on a partly saprophytized strain. If hung in an unsealed tube, even then, in spite of the free interchange of air, some slight inhibition over and above that exercised by the open tube is in evidence. The behavior of a strain of *Bacillus abortus* over 4 years under cultivation is illustrated by the following experiment.

Dilutions of a loopful of the turbid condensation water of a washed-down growth of 1/1, 1/25, 1/300, 1/7,500, and 1/90,000 in bouillon were prepared and a loopful transferred to two series of agar slants, one series (*a*) unsealed, the other series (*b*) provided with a tube of N NaOH and sealed. The dilution of 1/1 developed within 24 hours in both series. In the open series (*a*) the second dilution developed in 28 hours, in the (*b*) series in 48 hours, the third dilution of the (*a*) series in 48 hours, in (*b*) series in 72 hours. The fourth dilution in (*a*) appeared in 48 hours, in (*b*) in 4 days. The fifth in (*a*) appeared in 3 days, in (*b*) in 5 days.

The absence of CO_2 in a sealed tube thus inhibits growth of fresh strains even when large numbers of bacteria are transferred. It delayed growth of an old culture growing readily in open and sealed tubes in all dilutions from 1 to 2 days according to dilution when the tubes containing NaOH were sealed.

The next step was to test the effect of different concentrations of CO_2 on freshly isolated strains. To do this the following method was used.

Agar slants were inoculated each with a loopful of various dilutions and placed in Novy jars or desiccators. In all cases sealed controls of all dilutions were made. The CO_2 obtained from a commercial bomb was allowed to flow into a gas tank and air blown in until the desired volume dilution was secured. Before use a short time was allowed for a thorough mixture of the air and CO_2. Higher dilutions

were prepared by mixing lower dilutions with air. The mixture of CO_2 and air was allowed to flow through the jars for 4 or 5 minutes, the stop-cocks closed, and the diffusion allowed to go on for another 4 or 5 minutes. The procedure was repeated about six or seven times. In some experiments the air was partially exhausted, replaced by the CO_2 mixture, and the procedure repeated once or twice.

It is not necessary to go into the details of the various trials. They were begun with 10 per cent CO_2 and the amount of this gas gradually reduced to 0.1 per cent. In the different concentrations of CO_2 down to 0.1 per cent the growth of *Bacillus abortus* was prompt and vigorous. The maximum growth was usually obtained in 2 or 3 days. In 0.1 per cent CO_2 there was a slight delay of perhaps $\frac{1}{2}$ day. As stated above, control sealed tubes were prepared in all trials and in these the appearance of the film was delayed from 1 to $2\frac{1}{2}$ weeks or else completely suppressed. Among the many trials the following may be cited as an illustration.

Jan. 3, 1924. A guinea pig inoculated with contents of the fourth stomach of a fetus.

Feb. 13. Guinea pig chloroformed and cultures made with bits of spleen tissue on agar slants and the tubes sealed.

Feb. 20. Colonies of *B. abortus* have appeared.

Feb. 25. A subculture (plain agar) is heavily seeded from one of the spleen tubes and sealed.

Feb. 27. The film of growth is washed down with the condensation water and the turbid suspension used for dilutions in bouillon. A standardized loop was used and a loopful of the turbid condensation water considered as unity. Thirteen dilutions were made in bouillon, the last representing one 81-millionth of the original loopful.

One series was sealed and incubated. Of the other only the seven last dilutions, beginning with a dilution of 1/22,500 of the original loopful, were prepared and this set placed in a desiccator into which a mixture of air and CO_2 was driven containing 0.2 per cent CO_2.

Of the sealed tubes only the original tube receiving an entire loopful of condensation water and the tubes receiving each a loopful of the four succeeding dilutions developed, the first in 2 days, the last (1/300 dilution of the original loopful) in 10 days, the others in between these extremes. The rest failed to develop.

The higher dilution cultures in the CO_2 mixture beginning with the seventh dilution (1/22,500) all developed, the three first in 3 days, the others in 4 days.

In all the experiments with CO_2, next to the rapidity of growth the vigor of the films and colonies was in striking contrast to the feeble growth in sealed tubes.

In addition to the tests with CO_2, mixtures of nitrogen and air, as well as hydrogen and air, were tried, the trace of CO_2 having been removed from these mixtures. When growth in the control sealed tubes was evident after 5 to 7 days, none had occurred in the mixtures. CO_2 was then passed in to make 2 per cent by volume. In all tubes growth appeared within 2 days.

A limited number of experiments were made with mixtures of air containing higher percentages of CO_2. In mixtures containing 90 per cent CO_2 the appearance of films was slightly retarded. In an atmosphere of 100 per cent CO_2 four dilutions of 1/25 to 1/1,800 loopful were cultured. Of these the first dilution showed growth in 8 days, the second in 10 days. The jar was opened after 14 days. The fourth dilution (1/1,800) was without growth; the third had one colony.

In an atmosphere of 10 per cent CO_2 in 90 per cent N the growth was nearly as prompt and rich as in air and 10 per cent or lower volumes of CO_2. In a mixture of 90 per cent H and 10 per cent CO_2 all dilutions (up to 1/1,200) developed luxuriously within 4 days. There may have been traces of oxygen in the H, N, and CO_2 gases to account for the growth in the presumed absence of O. These preliminary tests indicate that with a proper CO_2 supply, the organisms will thrive on traces of oxygen.

Thus far the descriptions have applied to tubes of sloped agar. Plate cultures fail to develop when made in the usual way. Placed in jars in an atmosphere containing CO_2 the appearance of colonies is prompt and no suppression is in evidence. The figures given in the foregoing pages were obtained by plating dilutions and exposing them to an atmosphere containing CO_2.

When bacteria are distributed equally through melted agar and the tubes promptly chilled in a deep layer a film of colonies appears at the surface in tubes sealed. Tubes left unsealed remain free from growth. When such tubes are placed in air containing CO_2 a film appears on the surface and not below.

The repressing and inhibitory effects of drying of cultures have been cited by authors as perhaps accounting for the stimulating effect of CO_2 in closed vessels. Drying is undoubtedly a factor but it does not account for the behavior of the tubes in a CO_2 atmosphere.

Inoculated unsealed agar tubes up to 11 days old, which had failed to grow and had lost their condensation water by drying, were promptly covered with colonies when placed in jars containing CO_2. Tubes older than this could not be revived. Tubes placed in jars containing ordinary air full of moisture failed to develop.

Some authors have claimed that the delicate adjustment of freshly isolated strains to a given hydrogen ion concentration may account for the action of CO_2 in bringing about the proper pH. Inasmuch as this adjustment involves not only the pH range but also the nature of the ions used, only a few preliminary experiments were made in view of a broader inquiry into this aspect of the question.

The pH range within which *B. abortus* from freshly isolated strains may multiply in sealed tubes was determined by adding acids and alkalies to the ordinary agar of about pH 7.0. Normal phosphoric acid and normal sodium hydroxide were added in calibrated drops to the melted agar and the latter sloped as it cooled. On the acid side the limit of multiplication was reached when 0.16 cc. of N H_3PO_4 was added to 6 cc. of agar, lowering the pH to about 6.0 or 6.2. With N NaOH the limit was reached when between 0.04 and 0.08 cc. was added to 6 cc. agar, raising the pH to about 7.8 or 8.0. When the tubes were heavily seeded the range was in some tests slightly broadened to the next unit drop of 0.04 cc. N acid or alkali. When a tube of N NaOH was suspended in a tube of sterile agar and the tube kept sealed and incubated for a week the rise of the pH was from 0.1 to 0.3 only.

In view of the relatively broad pH zone within which *Bacillus abortus* multiplies in sealed tubes and the suppression of multiplication in the presence of a CO_2 absorbent, with but a slight change of pH, the contention that CO_2 simply adjusts reaction does not apply to *Bacillus abortus*.

When a series of tubes with additions of acid and alkali were incubated in the presence of 2 per cent CO_2 a very prompt and vigorous multiplication took place in all tubes of the range given above with a slight extension of growth in the alkaline range. Evidently the wide range of CO_2 concentrations, from 0.1 to 10 per cent, which was found to eliminate inhibition and suppression of multiplication and also to stimulate growth quantitatively militates against the assumption that this gas merely regulates the hydrogen ion concentration.

DISCUSSION.

The foregoing experiments carried out on a series of over 50 fresh cultures of *Bacillus abortus* during the past 2 years offer a method for testing the saprophytism of the strains. This in its extreme state consists in a relative indifference to free contact with the air and to the absence of free CO_2. The significance of CO_2 to the recently isolated culture is not cleared up by these culture tests. It may act as a catalyst, or coenzyme, or it may be used as a source of carbon in the manner first pointed out by Winogradsky in 1890 for the nitrifying bacteria and more recently confirmed by Meyerhof[23] for the organisms oxidizing nitrites to nitrates. The prompt growth in mixtures of 10 per cent CO_2 with N or H, the only ones tried, indicates that mere traces of O may suffice provided CO_2 is also available. When the latter is removed neither air nor mixtures of N or H with air permit multiplication.

Growth in mixtures of air containing as much as 90 per cent CO_2 was less rapid than in mixtures of 10 per cent or less, but in the end as vigorous and without suppression of high dilutions. In 100 per cent CO_2 high dilutions were suppressed; the lower dilutions throve, probably on traces of O still present.

On several occasions as a result of faulty technique, one or two colonies of moulds appearing in a high dilution culture caused the appearance of a large number of uniformly distributed colonies late when no growth was expected and when lower dilutions remained free from growth. The uniform size and distribution of such colonies suggests the influence of a gaseous element rather than one working through the agar medium.

The behavior of the bacillus in the sealed tube may be explained tentatively by the production of CO_2 by the transferred bacteria which is then retained by the seal. The favoring action of mass inoculations in starting the culture harmonizes with this view. The same is true of the occasional appearance of single colonies when millions of bacteria have been transferred. The single bacillus has been kept alive by the respiration of many perishing bacilli. The appearance of crops of colonies is a further support of this view.

[23] Meyerhof, O., *Arch. ges. Physiol.*, 1916, clxiv, 353.

A few scattering colonies appear and become 1 mm. in diameter. Suddenly a second much larger crop appears over the entire agar slope. Even a third may appear. The older saprophytized strains may require CO_2, but if so the requirement is masked by the early CO_2 production of the culture itself.

The behavior of *Bacillus abortus* in tubes of bouillon does not lend itself as well to quantitative studies bearing on the rate of multiplication. The organism is however favorably influenced by the presence of CO_2 as compared with the unsealed and sealed tube. The difficulty in establishing a focal mass effect to favor individual bacteria to multiply owing to convection currents and constant dilution may account for the conditions observed.

Further studies will determine whether *Bacillus abortus* stands alone in its relation to CO_2 or whether some other pathogenic types share this requirement. If they do the isolation and early cultivation of such types should be greatly promoted by its use. It is to be borne in mind that slight changes of environment may lead to failure or success if CO_2 has the significance for them that it has for *Bacillus abortus*. For instance, cultures of strains sensitive to CO_2 placed in a crowded incubator closed for 1 or 2 days may multiply, whereas another set in an empty incubator may fail to grow.

If we permit our imagination to play for a moment we may discern a certain parallel between the phenomena described and the process of infection. If we regard the agar substrate as the invaded host we have the outbreak of disease (or growth) depending on the dose in the first place. A large dose may overcome the unfavorable equilibrium. If the dose is too small, the invading organism fails to multiply, becomes latent until changes occur favorable to it. If the change comes soon (CO_2), a retarded attack (growth) may result. If beyond a certain period, the organism may have died.

CONCLUSIONS.

The relative absence of saprophytism in freshly isolated strains of *Bacillus abortus* is conveniently measured by inoculating a series of agar tubes with successive dilutions of culture and sealing the tubes. The appearance of films of growth is delayed from 3 to 17 days and suppressed with the increase in the dilutions. As many

as 100,000 bacteria per sq. cm. of agar surface are suppressed. When the confined air contains CO_2 in a concentration as low as $\frac{1}{4}$ per cent and up to 10 per cent, the inhibition and suppression do not occur. Concentrations down to 0.1 per cent are still capable, even though in a slightly retarded manner, of bringing high dilutions into growth. In all cases the resulting growth was remarkably vigorous when compared with that in sealed tubes.[24]

[24] After completion of the manuscript, an article on porcine abortion by Good and Smith was brought to my attention (Good, E. S., and Smith, W. V., *J. Bact.*, 1916, i, 415). The strain of *B. abortus* isolated grew readily in air.

A COMPARATIVE METHOD FOR TESTING THE ENZYMES OF LIVING HEMOLYTIC STREPTOCOCCI.

I. LIPASE.

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(Received for publication, April 10, 1924.)

INTRODUCTION.

During the study of a series of cases of acute extensive superficial gangrene in which a hemolytic streptococcus was constantly present and seemed to be the causative agent, it was observed that the organism had a very great affinity for the subcutaneous fat. The infection invariably spread rapidly in this tissue and caused an extensive necrosis and solution of the fatty tissue. This observation led to an investigation of the enzymes of the streptococci recovered from these cases.

It is generally believed that bacteria carry on their metabolic processes by the aid of ferments which act upon the surrounding medium and prepare it in a suitable manner for ingestion. These enzymes may be liberated during the life of the bacteria or upon their death and dissolution. It has been frequently shown that these enzymes are capable of digesting carbohydrates, proteins, and fats (10). The methods which have been described, however, are not adapted to strict quantitative estimations. The recent methods which Stevens and West (9) used in their investigation of the enzymes of hemolytic streptococci are not available for a quantitative study because of the difficulty of completely extracting the enzyme by the grinding process.

Because of the complete solubility of the pneumococcus in bile, Avery and Cullen (1) were probably able to get all of the endoenzymes of that organism into solution. So far, this has not been possible with the streptococcus. We have tried various chemical substances and physical forces in an attempt to find a method that would completely liberate the enzymes without destroying them. All of these efforts were unsuccessful. We therefore discarded the methods of extraction of enzymes from dead organisms and turned to the more logical study of living organisms. It was observed that living streptococci suspended in

water showed a marked lipolytic activity in the presence of ethyl butyrate. It was then found that streptococci, in common with other organisms, die off rapidly when suspended in water. This led to the study of suspending fluids which would maintain the streptococcus for a considerable length of time without increase or diminution of numbers. One of us with Zau (8) found that the hemolytic streptococcus will survive for a week or more in dilutions as high as 100 organisms per cc. in three solutions; namely, Locke's solution, 0.2 per cent sodium citrate, and a balanced solution containing 1.0 per cent sodium chloride and 0.05 per cent calcium chloride, if to each of these solutions 0.1 per cent gelatin be added. Certain other solutions containing 0.1 per cent gelatin, including distilled water, will maintain the streptococci for several days. These gelatin solutions were therefore considered to be available for quantitative determinations. Gates (6) has devised a fairly accurate method of obtaining standard suspensions of bacteria so that in the gelatin solutions different organisms may be quantitatively compared.

The usual method of determining lipolytic activity by other workers has been to titrate the acid produced in the enzyme substrate mixtures with sodium hydroxide. Dietz (3) has shown, however, that enzyme preparations of different activity, as measured by their velocity constants, reach the same equilibrium point if allowed to continue to completion. Therefore, if sufficient time be allowed, the amount of titrable acidity produced will be the same, even if the concentration of the enzyme is varied. Within certain limits, when there is an excess of substrate present in an enzyme-substrate system, the velocity of the reaction is in direct linear proportion to the quantity of active enzyme present. We have found this to be true of preparations of pancreatic lipase and ethyl butyrate. These facts, together with the difficulty of titrating the minute quantities of acid produced by small quantities of bacteria, suggested the possibility of measuring the velocity of the reaction between certain definite hydrogen ion concentrations.

EXPERIMENTAL.

The organism used in these experiments was obtained from a case of acute gangrene of the leg, one of a series of cases previously reported (7). It was a typical *Streptococcus pyogenes* in its cultural characteristics. It was kept as a stock culture in horse blood broth in which it would survive for 2 months without transfer. During

these experiments it was kept actively growing by frequent transplantation. 0.1 cc. of this stock culture was planted in 25 cc. of 1 per cent dextrose liver digest broth in a 50 cc. centrifuge bottle and incubated for 12 to 14 hours. After incubation the culture was centrifuged, washed twice, and then taken up in the suspending fluid to be used for the test. The concentration was then determined by the method of Gates (6) and adjusted so that 1 cc. contained approximately 5 billion cocci. Three standard color tubes were prepared by boiling 6 cc. of the standard suspension for 1 minute, dividing this into three parts, centrifuging, and taking up the sedimented bacteria from each part in 2 cc. of Clark's (2) standard buffer mixtures at pH levels of 8.0, 7.6, and 7.2. These suspensions were placed in 5 cc. test-tubes and 0.05 cc. of phenol red was added to each tube as indicator. Thus each standard color tube had the same relative turbidity as the suspension of living organisms to be tested. With the suspended organisms, the colors of the standard color tubes were slightly paler than the buffer solutions alone but they remained constant for several days. 2 cc. of the living suspension to be tested were placed in a dry sterilized 5 cc. test-tube. Corks were used instead of cotton plugs in order to prevent gaseous exchange. 0.05 cc. of phenol red was added, then 0.2 cc. of ethyl butyrate and enough $\frac{N}{50}$ sodium hydroxide to bring the mixture, after shaking, to a color slightly more alkaline than the pH 8.0 standard color tube. This usually required 0.2 cc. of the alkali. In later tests the alkali was added first and the ethyl butyrate afterward in order to have the ethyl butyrate the last addition before the actual test began. Control tubes were set up of the standard suspension of organisms without ethyl butyrate and the suspending fluid alone with ethyl butyrate. All of the tubes were incubated in the water bath at 37.5° and shaken every minute to maintain contact between the bacteria and the substrate which otherwise promptly floated to the top of the fluid. After each shaking, the color of the fluid was compared with the standard color tubes and a time reading taken when the color was exactly the same. It was found that the colors could be much more accurately compared if the tubes were held in front of a frosted electric bulb covered by wet filter paper than if held up to the daylight. As acid formed in the "active tubes," the color passed through

the ranges covered by the standard color tubes, the rapidity of acid formation being considered indicative of the amount of lipase present, providing the controls were negative. A direct quantitative comparison was thus possible for the same organism under different conditions and for different organisms under identical conditions. The comparison could be made between the pH 8.0 and 7.6 levels or between the 7.6 and 7.2 levels or both together.

A preliminary experiment determined the fact that 0.2 cc. was an excess of substrate for the amount of lipase present. Tubes were set up with the same quantity of standard living suspensions but with increasing amounts of ethyl butyrate, 0.1, 0.2, 0.3, and 0.4 cc.

TABLE I.

Relative Speed of the Reaction in Different Suspending Media.

Tube No.	Suspending fluids.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
		min.	*min.*	*min.*
1	0.2 per cent sodium citrate with 0.1 per cent gelatin.	17	38	55
2	Locke's solution with 0.1 per cent gelatin.	18	40	58
3	1.0 per cent sodium chloride and 0.05 per cent calcium chloride with 0.1 per cent gelatin.	6	18	24
4	Twice distilled water.	8	22	30

Controls: Standard suspension without ethyl butyrate and suspending fluids alone with ethyl butyrate, all negative.

being used. In this series the passage time from pH 8.0 to 7.2 was 22, 22, 22, and 19 minutes respectively. For the subsequent tests 0.2 cc. was used. Tests were then performed to determine (1) the best suspending fluid for the reaction; (2) the effect of diluting the standard suspension; (3) the effect of varying the incubation time for the culture of the organism; (4) the optimum temperature for the reaction; (5) the effect of previous contact with higher temperatures; (6) the optimum hydrogen ion concentration; (7) the action of a strain when relatively avirulent in comparison with the same strain made relatively virulent for rabbits by repeated animal passages; and (8) the action of different strains of hemolytic streptococci from different clinical sources.

The Relative Activity in Various Suspending Fluids.

Since it had been found that the hemolytic streptococcus is well preserved in Locke's solution, in 0.2 per cent sodium citrate, 1.0 per cent sodium chloride with 0.05 per cent calcium chloride ("20:1" mixture), and even in water if 0.1 per cent gelatin be added to each (7), these four solutions were tested. It was found that with "20:1" mixture the results were attained more quickly and the color changes were more clear-cut than with the other solutions. The result is shown in Table I. The "20:1" mixture was used in all of the subsequent tests.

The Effect of Dilution of the Organisms.

The test was set up with five tubes, the first of which contained the standard suspension with approximately 5 billion organisms per cc. The others contained 4, 3, 2, and 1 billion respectively. The dilutions were made with the "20:1"-gelatin suspending fluid. This made the higher dilutions less turbid than the standard color tubes. When the dilution was made with a suspension of heat-killed organisms to make the turbidity the same, the relative result was the same.

The results are given in Table II. The curve in Text-fig. 1 indicates that the rapidity of the reaction is approximately in linear proportion to the concentration of active substance, at least for the three highest concentrations.

The Relative Action of Young and Old Cultures.

0.1 cc. of the stock culture was incubated overnight in 25 cc. of 1 per cent dextrose liver digest broth. In the morning after 14 hours incubation, 2 cc. of this culture were transplanted to another tube containing 25 cc. of media. Incubation was continued until the afternoon when the test was made for lipase activity. At that time the first culture had been incubated for 21 hours and the second for 7 hours.

The 7 hour subculture made the color change in 19 minutes while the 21 hour culture took 66 minutes. This very important finding will be further elaborated below.

The Optimum Temperature of the Lipolytic Reaction.

The test was set up with five tubes containing the standard suspension of living organisms, each one having two control tubes. The lipase activity was then tested at ice box temperature, 15° C.; room temperature, 27°; and water bath at 37.5°, 50°, and 62°.

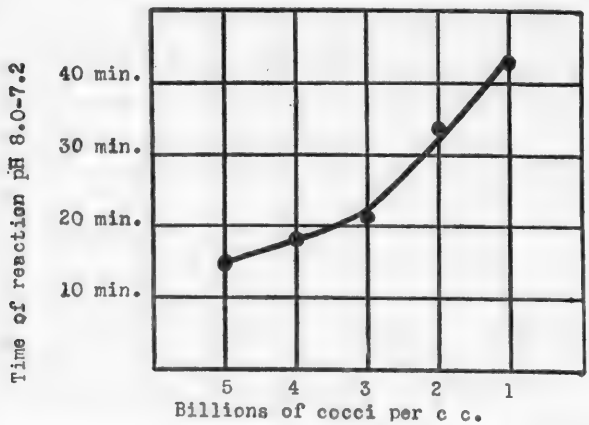
TABLE II.

Effect of Dilution of the Organisms on the Rapidity of Lipase Action.

Suspending fluid "20:1"-gelatin mixture (1.0 per cent sodium chloride and 0.05 per cent calcium chloride with 0.1 per cent gelatin).

Tube No.	Estimated No. of cocci per cc.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
		min.	*min.*	*min.*
1	5 billion.	4	11	15
2	4 "	5	13	18
3	3 "	6	15	21
4	2 "	9	25	34
5	1 "	14	29	43

Controls: Standard suspension (approximately 5 billion cocci per cc.) without ethyl butyrate and "20:1"-gelatin solution with ethyl butyrate both negative.



TEXT-FIG. 1. Curve representing the effect of dilution of the organisms on the rapidity of lipase action.

The optimum temperature was found to be 37.5°. At 50° the control containing the suspending fluid and ethyl butyrate changed slightly, while at 62° this control tube passed through the whole zone from pH 8.0 to 7.2 faster than the suspension of organisms with ethyl butyrate. The results are shown in Table III and the curve for the reaction in Text-fig. 2.

TABLE III.

Optimum Temperature for the Lipase Action.

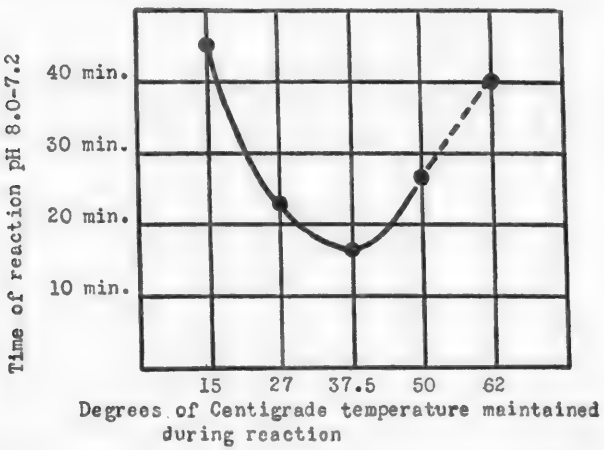
Suspending fluid "20:1"-gelatin mixture.

Tube No.	Temperature.	Contents of test-tubes.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
	°C.		*min.*	*min.*	*min.*
1	15	Standard suspension and substrate.	19	26	45
2	15	" " alone.	—	—	—
3	15	"20:1"-gelatin solution and substrate.	—	—	—
4	27	Same as No. 1.	11	12	23
5	27	" " " 2.	—	—	—
6	27	" " " 3.	—	—	—
7	37.5	" " " 1.	7	10	17
8	37.5	" " " 2.	—	—	—
9	37.5	" " " 3.	—	—	—
10	50	" " " 1.	14	13	27*
11	50	" " " 2.	—	—	—
12	50	" " " 3.	26	—†	—
13	62	" " " 1.	14	26	40‡
14	62	" " " 2.	—	—	—
15	62	" " " 3.	8	27	35

* Cannot be said to be entirely enzyme action because of slight change in control tube.

† Did not reach pH 7.2 by end of experiment (1 hour).

‡ Cannot be said to be enzyme action because of more rapid change in the control tube with ethyl butyrate.



TEXT-FIG. 2. Curve representing the optimum temperature for lipase action. Dotted line represents uncertainty of enzyme action because of changes in control tubes.

The Effect of Previous Contact with Higher Temperatures.

The test was set up with five tubes containing the standard suspension of organisms. The tubes were then subjected to temperatures of 37.5°, 40°, 60°, 80°, and 100°C. respectively for 10 minutes. They were then cooled and the test for lipase activity carried out with the proper controls in water bath at 37.5°. It was found that the tubes previously heated to 60°, 80°, and 100° made the change from pH 8.0 to 7.6 very slowly but never reached 7.2, while the other two tubes promptly passed on to the acid end of the color range. The test was then repeated between the active and inactive levels. Five portions of the standard suspension were heated for 10 minutes at 40°, 45°, 50°, 55°, and 60°C. respectively. After heating, 0.1 cc. from each tube was cultured in plain broth to determine the viability of the organisms. The lipolytic activity of the heated suspensions was then tested.

TABLE IV.

Effect of Previous Contact for 10 Minutes with Higher Temperatures.

Suspending fluid "20:1"-gelatin solution.

Tube No.	Previously subjected temperature for 10 min.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
	°C.	*min.*	*min.*	*min.*
1	37.5	14	18	32
2	40	18	22	40
3	60	75	*	
4	80	68	*	
5	100	60	*	

Controls: Standard suspension (5 billion cocci per cc.) without ethyl butyrate negative; "20:1"-gelatin solution with ethyl butyrate negative.

* Did not reach pH 7.2 by end of experiment (3 hours). Initial change manifestly not due to enzyme action.

It was found that the suspensions that had been heated to 40°, 45°, and 50° were about equally active, while the action of the suspension heated to 55° was markedly delayed and the suspension heated to 60° was practically inactive. Correspondingly the cultures of the heated organisms showed profuse growth from the suspensions heated to 40°, 45°, and 50°, while from the suspension heated to 55° out of approximately 500 million organisms planted, only six to eight colonies were found clinging to the sides of the test-tube. When shaken up and incubated longer they grew out profusely. The suspension heated to 60° was sterile. The results of these tests are shown in Tables IV and V and the curve in Text-fig. 3.

TABLE V.

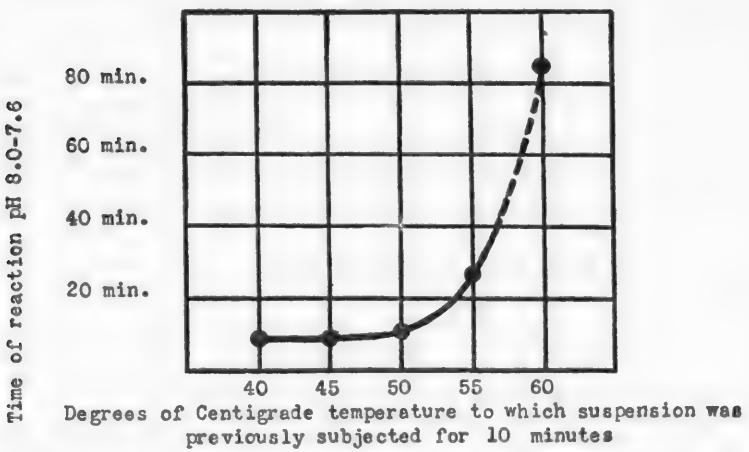
Effect of Previous Contact with Temperatures between 40° and 60° C. for 10 Minutes on the Lipolytic Activity and Viability of the Organisms.

Suspending fluid "20:1"-gelatin mixture.

Tube No.	Previously subjected temperature for 10 min.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.	Culture after heating
	°C.	*min.*	*min.*	*min.*	
1	40	9	15	24	Profuse.
2	45	9	15	24	"
3	50	11	15	26	"
4	55	27	37	64	6 to 8 colonies.
5	60	85	*		No growth.

Controls: Heated standard suspension without ethyl butyrate and suspending fluid alone with ethyl butyrate at each temperature. The controls at 60° gradually changed color, thus making enzyme action at this temperature questionable.

* Did not reach 7.2 by end of the experiment (3 hours).



TEXT-FIG. 3. Curve representing the effect of previous contact with heat on the rapidity of lipase action. Dotted line represents uncertainty of enzyme action because of slight changes in control tubes.

When the experiment was repeated and the heating process continued for 30 minutes at 55°C., the organisms were completely destroyed and there was no lipolytic activity, while at the three lower temperatures the viability of the organisms and the lipolytic action were not affected.

The Optimum Hydrogen Ion Concentration.

For this test, the speed of color change could not be used as an indication of acid production because a given amount of acid has more effect on the color changes in the higher numerical pH levels than in the lower pH levels. It was found necessary to start the reaction at standard color levels and after a period of time, titrate back to the standard color. The standard colors were made by suspending heat-killed organisms, in the standard concentration of approximately 5 billion cocci per cc., in Clark's (2) buffer solutions at the pH levels 5.4, 6.0, 6.6, 7.2, 7.8, 8.4, 9.0, and 9.6 and using the corresponding indicators. The standard suspension of living organisms was centrifuged in 4 cc. quantities, washed once, and then taken up in the same concentration with "20:1"-gelatin solution previously titrated to the levels indicated above. 2 cc. of the live suspension at each hydrogen ion level were used for the test. After adding the ethyl butyrate, the reactions were again adjusted to the levels indicated. Controls were set up for each hydrogen ion level containing standard suspension alone, suspending fluid alone, and suspending fluid plus ethyl butyrate. After incubation for 45 minutes, enough $\frac{N}{100}$ sodium hydroxide was added to each tube to bring the color back to the standard. No change occurred in the tubes at pH 5.4 and 6.0. At pH 9.0 and 9.6 the controls changed to such an extent that the actual lipolytic effect was uncertain. Between pH 6.6 and 8.4 the slight changes in the control tubes could be adjusted by subtraction so that the relative amount of lipolytic action could be fairly accurately determined.

The optimum hydrogen ion concentration was found to be pH 7.8. The results are shown in Table VI and Text-fig. 4.

The Relation of the Virulence of the Organism to the Lipolytic Activity.

The strain used in all of the preceding experiments had been passed through three rabbits and had a minimal lethal power, on intravenous injection, of 1 cc. of a standard suspension estimated to contain 1 billion organisms per cc. The same strain was passed through thirteen rabbits, after which it killed repeatedly at 0.001 cc. of the standard suspension.

TABLE VI.

Optimum Hydrogen Ion Concentration for Lipolytic Action.

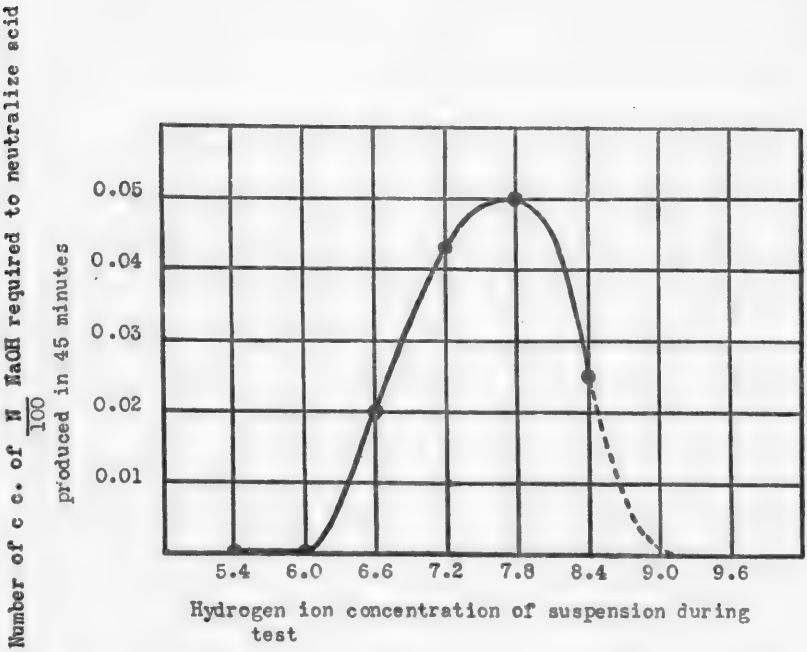
Suspending fluid "20:1"-gelatin mixture.

Tube No.		Levels of hydrogen ion concentration.							
		5.4	6.0	6.6	7.2	7.8	8.4	9.0	9.6
1	Standard suspension with ethyl butyrate.	0	0	0.25	0.7	0.95	0.9	+++	++++
2, 3, and 4	Summation of controls.	0	0	0.05	0.275	0.45	0.65	+++	++++
Difference (representing enzyme action).....		0	0	0.2	0.425	0.5	0.25	–	–

The tubes were set up with the colors exactly conforming to standard color tubes for each hydrogen ion level.

The figures represent the amount of $\frac{N}{100}$ NaOH required to bring the color of the mixtures back to the standard colors after 45 minutes incubation at 37.5°C. in the water bath.

Controls: Standard suspension without ethyl butyrate, suspending fluid with ethyl butyrate, suspending fluid alone.



TEXT-FIG. 4. Curve representing the optimum hydrogen ion concentration for lipase action. Dotted line represents uncertainty of enzyme action because of marked changes in the control tubes.

The lipolytic test was set up for standard suspensions of the virulent and the relatively avirulent organism in the usual way. The speed of the reaction of the two suspensions proved to be almost the same for both.

There was no proportionate increase of lipolytic activity with the increase in virulence and no indication that virulence in any way depends upon the lipolytic property. The figures are given in Table VII.

TABLE VII.

Comparison in Lipolytic Action between an Organism of Low Virulence for Rabbits and the Same Strain When in a Condition of Relatively High Virulence.

Suspending fluid "20:1"-gelatin mixture.

Tube No.	Virulence.	No. of rabbit passages.	Time required between pH 8.0 and 7.6.	Time required between pH 7.6 and 7.2.	Total time required between pH 8.0 and 7.2.
			min.	*min.*	*min.*
1	Low.	3	7	18	25
2	High.	13	9	20	29

Controls for each tube: Standard suspension of organisms without ethyl butyrate and the suspending fluid with ethyl butyrate all negative.

The Action of Different Strains of Hemolytic Streptococci Obtained from Various Clinical Sources (with Particular Reference to Strains Recovered from a Series of Cases of Acute Superficial Gangrene.)

The test has not been applied to a large series of cases but a direct comparison has been made between eighteen strains recovered from a definite clinical disease entity which has been studied by one of us and called hemolytic streptococcus gangrene (7) and strains from seven other sources including two from the throats of scarlet fever cases. Inasmuch as there seemed to be a great affinity between the organism and the subcutaneous fat in the gangrene cases, it was thought that this might be explained by an increase in lipolytic ferment. This theory is hardly tenable after studying the results of this test. Although there is more active lipase in the organisms of the gangrene group as a whole than in the group of controls, two of the latter are regularly more active than several of the former and there is no clear-cut difference based upon this property. The freshness of the strain is immaterial. Some strains which were kept in horse blood broth for over a year were just as active in the production of lipase

as those strains recently recovered from patients. The tests were done at three different times with certain of the controls used in all three tests. The organisms were planted, first in 5 cc. of plain broth, and after 10 to 12 hours incubation 2 cc. were transferred to 1 per cent dextrose broth. The periods of incubation and standardization of suspension were kept as uniform as possible. It may be that in a large series of cases it will be possible to make some biologic grouping on this basis.

For the present all that can be said is that the activity of lipolytic ferment does not appear to correspond to any specific disease-producing function.

DISCUSSION.

The delay in color change from pH 8.0 to 7.2 in the gelatin-citrate and gelatin-Locke's solutions employed in the first set of tests (Table I) is probably due to the fact that these solutions have more buffer action than the "20:1"-gelatin mixture. The citrate anion is a buffer and the Locke's solution contains a small amount of sodium bicarbonate. The slower action of gelatin-water may be due to the poorer preserving action of that fluid. It was thought possible that in the gelatin solutions some of the acid formed was due to the action of a gelatinase. On the other hand, it was recognized that gelatin itself has a buffer action. When the test was repeated with the final suspension of the organisms in one tube in "20:1" mixture *with* gelatin and in the other *without*, the color change was definitely more rapid in the solution without gelatin. This shows that if there is any acid formed from the gelatin it is not sufficient to counterbalance the buffer action of the gelatin itself.

The effect of the dilution of the organisms on the lipase action is about what would be expected. The margin of experimental error may be enough to explain why the curve is not a straight line. Or it may be that the prolonged contact with the products of the reaction, in spite of their extreme dilution, delays the reaction. This point is considered below.

The most significant finding in the whole series of experiments has been the great activity of young cultures. This led to a more thorough investigation of the factor of the length of incubation.

Was the fat-splitting a function performed only by young, rapidly multiplying, metabolizing bacteria? In what phase of growth was the lipase action most rapid?

At 8 p.m., 0.1 cc. of the stock culture was planted in two centrifuge bottles each containing 25 cc. of 1 per cent dextrose liver digest broth pH 7.6. At 4 a.m., 2 cc. of this 8 hour culture were transferred to each of twenty similar bottles of media. At 4 hour intervals for a period of 28 hours certain of these cultures were taken and the following tests carried out on them.

(*a*) Hydrogen ion concentration of the supernatant fluid.

(*b*) Concentration of organisms in the media. The total number was determined by the Gates (6) turbidimeter method and that number divided by the number of cubic centimeters of media used. This estimation was only approximate because there were not exactly 25 cc. of media in each bottle.

(*c*) The viability of the organisms. A standard suspension estimated to contain 1 billion cocci per cc. was made by means of the Gates (6) turbidimeter in the "20:1"-gelatin mixture and a series of seven dilutions made, each one containing a tenth of the number in the previous dilution. The final dilution was, therefore, estimated to contain 100 organisms per cc. 5 cc. of broth medium were then added to 1 cc. of each dilution and the whole series incubated. The results were read 24 hours after the 28 hour test was completed.

(*d*) The lipolytic action of the organisms suspended in the "20:1"-gelatin solution.

The various steps in the tests were done at precisely similar periods after incubation ceased. For the 4, 8, and 12 hour tests, six, four, and three bottles of culture were respectively used. For the next three tests two bottles were used. For the 28 hour test one was used.

The results of these tests are shown in Table VIII and Text-fig. 5. The significant points brought out by these tests were the following.

1. In glucose broth the hydrogen ion concentration reached a point of equilibrium at pH 5.1 in 16 hours.

2. Multiplication was rapid up to 8 hours but gradually slowed up and after 16 hours entirely ceased.

3. After 12 hours the organisms began to die off rapidly and at 24 hours none survived in a suspension containing 100 million organisms per cc.

4. The lipase action was rapid and constant as long as the organisms were actively multiplying but became slower as multiplication became slower, although the organisms were still viable. When the organisms were all dead the lipase action reached another constant five or six times as slow as that of the actively multiplying organisms.

The slow action may represent the action of an endoenzyme while the rapid action appears to be a function of the living organism. It may be an ectoenzyme. It is evidently not enough that the organisms be alive but they must be in an actively multiplying growth phase. Foster (5) has shown that the acid curve for hemolytic streptococci in a dextrose broth medium depends upon a number of factors; namely, the initial hydrogen ion concentration of the medium, the age of the parent culture, and the amount of the inoculum. He

TABLE VIII.

Effects of a Varying Duration of Incubation on the Hydrogen Ion Concentration of the Medium and Concentration and Viability of Organisms and the Lipase Activity.

Suspension fluid for the viability tests and lipase reaction "20:1"-gelatin mixture.

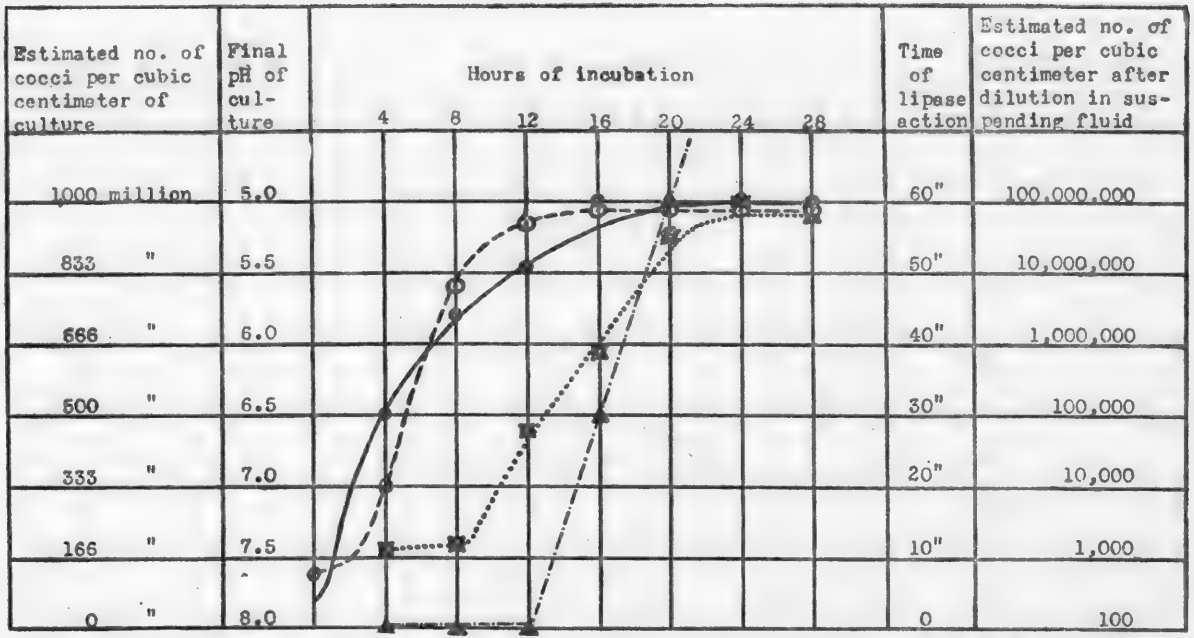
Duration of incubation.	pH level reached.	Concentration of organisms per cc. of culture medium.	No. of cocci per cc. in highest viable dilution.	Total time taken for lipase action pH 8.0–7.2.
hrs.				*min.*
4	7.0	500 million.	100	11
8	5.6	748 "	100	12
12	5.2	848 "	100	28
16	5.1	1,028* "	100,000	39
20	5.1	928* "	100,000,000	56
24	5.1	1,026 "	∞	60
28	5.1	1,028 "	∞	58

Hydrogen ion concentration determined by comparator method.

Lipase action determined in the usual way in "20:1"-gelatin suspending fluid.

* This discrepancy is probably due to the fact that the quantity of media in the tubes was not exactly 25 cc. The Gates method also has slight experimental error.

did not have a suitable method for determining the viability of the organism, for, as he remarks, the plating method is unreliable. Therefore, he could not fully appreciate the factor of viability. If dextrose broth is used, the incubation of the parent culture should not be over 8 hours and the subculture time should be the same, in order that one may be sure that the organisms are all alive and actively growing. It is obvious that these factors must all be constant



TEXT-FIG. 5. Curves representing the effects of varying duration of incubation in 1 per cent dextrose liver digest broth. A few organisms grew out in the highest dilutions of the 16 hour culture when incubated from 36 to 48 hours. Not grossly apparent before 36 hours.

⊙-------⊙ Hydrogen ion concentration of medium after incubation for the hours indicated.

●——— Estimated number of cocci per cubic centimeter of medium after incubation for the hours indicated.

■.....■ Time taken for lipase action color change pH 8.0 to 7.2 after incubation for the hours indicated.

▲—·—·—▲ Highest dilution at which organisms were viable after incubation for the hours indicated. Readings made 24 hours after 28 hour test.

for any test involving a comparison of different strains. The time variations in all of the tests presented in the first part of this paper, are attributable to a variation in these factors for the fact had not been recognized when they were done that the organisms begin to die off after 12 hours. Most of the tests were carried out 12 to 14 hours after inoculation with 0.1 cc. of the stock culture. With this small amount of inoculum, the period at which death began was probably somewhat later than in the test presented above, in which 2 cc. of an 8 hour culture were used as the inoculum. In the early test which compared a young with an old culture, the inoculum for the 7 hour culture had been incubating for 14 hours and was probably not as active as at an earlier period. However, the facts brought out by the tests still stand, because the preliminary steps in each test were the same and the conditions were changed only after the standard suspensions had been made. Thus the relation of the varying conditions is the same as it would be under more ideal cultural conditions. The time of the reaction is slower, in general ranging between 20 and 30 minutes. The tests with different strains were all repeated with 8 hours incubation of both the parent culture and the subculture. With this short incubation, the virulent and avirulent strains produced their effects in 11 and 10 minutes respectively. The gangrene strains did not show a clear-cut increase of lipolytic activity over that of certain of the control strains.

It was thought that if the enzyme were an ectoenzyme, it might be given off into the medium in an old culture. It was found, however, that the supernatant fluid of an old culture had no demonstrable effect on ethyl butyrate. It may be that the buffer action of the medium concealed a slight action. There was no difference to be found between fresh and supernatant broth. Avery and Cullen (1) were not able to find any lipolytic action in the filtrate from a pneumococcus culture and concluded that that organism did not give off the enzyme. From these tests it is apparent that the living organisms must be in contact with the substrate in order that they may produce the active lipase.

The fact that the optimum temperature for the lipolytic action lies in the neighborhood of the optimum temperature for the growth of streptococcus seems to argue in favor of the lipolytic action being a function of actively growing metabolizing organisms.

The effect of previous contact with heat on the lipolytic action tends to confirm the idea that this action is a function of living organisms, for the thermal death point of the streptococcus coincides quite definitely with the cessation of lipase action whether the heating be continued for 10 or for 30 minutes.

The optimum hydrogen ion concentration for the lipolytic action also conforms to the level of alkalinity favored by the streptococcus for most rapid growth (Fennel and Fisher (4)).

In the course of a number of the lipase tests an important fact was observed which should be presented here; namely, that the streptococcus is killed by the products of the ethyl butyrate digestion. Cultures made at 5 minute intervals during one test were sterile after the test had run between 25 and 30 minutes. At this time, the pH was slightly above 7.2 which was reached in 30 minutes. This is well above the acid death-point of the streptococcus in ordinary media and there was no death and no color change in the control tube containing organisms in suspension without ethyl butyrate. Thus it is seen that the organisms are killed by the products of the reaction rather than by the mere property of acidity. Therefore it is important not to start the test at a pH more than a fraction above pH 8.0 for if it is much higher, the digestive products may kill the organisms and thus delay the reaction. For this reason, the interval between pH 8.0 and 7.6 is probably more representative of the action of the living organisms than the interval between 7.6 and 7.2. It is in the nature of pH values for more acid to be required to accomplish the color change from 7.6 to 7.2 than to bring about that from 8.0 to 7.6 but in certain tests the delay may have been partly due to the death of some of the organisms. The presence of a buffer delays the color change but may not affect the toxic action of the digestive products. For this reason the "20:1" mixture has a decided advantage over the citrate solution and Locke's solution. The facts here presented all tend to confirm the idea that the rapid lipase action is a function of living, actively metabolizing organisms.

Although the organisms from the gangrene series do not show any greater activity in the splitting of ethyl butyrate than the control strains, the fact that a rapid lipase action is a function of rapidly multiplying organisms suggests another possible explanation for the

rapid necrosis and solution of the subcutaneous fat in these cases. It is possible that because of some biological relationship between these organisms and the invaded body, at present not understood, there is in these cases an unusually rapid multiplication of the organisms in the body or else they are not killed off as rapidly in the body fat by the products of their metabolism, as other strains are. Such hypotheses may be extremely difficult to test. We have failed to get a necrosis of human fat outside of the body either by injecting the organisms into the center of a piece of fat and then incubating or by adding a piece of fat to ordinary broth media and then inoculating with the streptococcus.

CONCLUSIONS.

1. A method is presented for the quantitative comparison under various conditions of the activity of the lipolytic enzyme of the hemolytic steptococcus. The speed of acid production as shown in the color change from pH 8.0 to 7.2, when the living streptococcus is suspended in association with ethyl butyrate, is considered to be indicative of the amount of ferment elaborated by the organism.

2. The lipolytic action is a function of living, actively growing organisms such as are present in 4 to 8 hour cultures.

3. The speed of the lipolytic action is approximately in linear proportion to the concentration of the organisms.

4. The lipolytic action is most rapid at 37.5°C., slower at 50°, and absent at 62°.

5. The organisms are partially destroyed and the lipolytic action is markedly delayed by previous heating to 55°C. for 10 minutes. Both the organisms and the lipolytic activity are completely destroyed by continued contact with this temperature for 30 minutes.

6. Increasing the virulence of the organism for rabbits by repeated animal passage does not increase the lipolytic action.

7. The predilection of the hemolytic streptococcus for the subcutaneous fat in local streptococcus infection associated with extensive superficial gangrene cannot be explained on the basis of an increase of lipolytic ferment in the organisms recovered from these cases over that of heterologous strains of streptococci.

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STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

III. Agglutination and Absorption of Agglutinin with Streptococcus scarlatinæ.

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(Received for publication, April 2, 1924.)

Recent studies of the group of hemolytic streptococci associated with scarlet fever indicate that the individual strains are closely related antigenically. These organisms are present in the early stages of the disease in the throats of practically all individuals suffering from scarlet fever. In addition, the specific streptococcus has been isolated from the uterine secretions in an undoubted instance of puerperal scarlet fever, from the wound in a case of wound scarlet, and from the local lesion in an instance of scarlet fever due to infection of a burn. More recently we have been able to obtain this streptococcus from the infected milk and from patients in a milk-borne epidemic of scarlet fever. The apparent specificity of the organism and its constant association with the disease again bring into prominence the question of its etiological relationship to scarlet fever. The main objections of Jochmann to this etiological significance, namely that hemolytic streptococci are not present in all cases of scarlet fever, and that the type of streptococcus present is indistinguishable from hemolytic streptococci obtained from other pathological processes, can no longer be maintained.

The importance of a continued effort to decide the significance of this association of streptococcus with scarlet fever is obvious. We have therefore continued the studies just mentioned with the hope of discovering additional evidence. It is probably of fundamental importance to determine beyond question the specificity of the type of streptococcus found in scarlet fever. Up to the present time chief reliance has been placed upon the agglutination reaction as

a test of specific serological relationship. This reaction while serviceable is especially likely for a variety of reasons previously discussed to furnish questionable results when used in the study of *Streptococcus hæmolyticus*. The low virulence of the streptococcus for test animals interferes with cross-protection experiments so that this method cannot be used. In the present study we have again employed the agglutination reaction and have attempted to confirm the results so obtained by determining the capacity of the strains under observation to absorb agglutinin specifically.

Material and Methods.—The strains of *Streptococcus hæmolyticus* used in the present study were collected from a variety of widely separated localities. The majority were obtained from the throats of patients in the scarlet fever wards of the Willard Parker Hospital, New York. A number of strains came from an outbreak of scarlet fever due to infected milk. This series includes a strain obtained from the suspected milk supply. In addition a small number of cultures from Copenhagen, San Francisco, Chicago, and Baltimore were studied. The cultures were obtained for the most part during the first few days of the disease. Hemolytic streptococcus was present on the plates from the throat secretions in 87.5 per cent of the individuals examined. This incidence figure is somewhat lower than that of Bliss (100 per cent) owing probably to the fact that some of the cultures were made later during the course of the disease than is desirable. Previous studies have shown that hemolytic streptococcus is only constantly demonstrable when an examination is made during the first days of an attack of scarlet fever. The technique of cultivation and agglutination was the same as that previously described for *Streptococcus hæmolyticus* by Dochez, Avery, and Lancefield and by Bliss.

The immune serum used in these experiments was prepared by inoculating rabbits intravenously first with heat-killed and then with living broth cultures. The serum of each animal was tested for the presence of natural agglutinins with two scarlatinal and two non-scarlatinal strains of streptococcus before immunization was begun. Rabbits possessing natural agglutinins for streptococcus were discarded. The first two series of inoculations were with amounts of heat-killed broth culture varying from 0.5 to 4.0 cc.

Each series consisted of four inoculations and an interval of 4 days was allowed to elapse between courses. The third and fourth series consisted of similar inoculation with living 18 hour broth cultures. If the animal lost weight during the process of immunization it was necessary to decrease the dosage. About 10 days after the last inoculation the animals were bled and the agglutinin titer of the serum determined. The agglutinins in antiscarlatinal sera tend to deteriorate rapidly and this deterioration is greatly accelerated by inactivation at 56°C. The most clear-cut reactions are obtained when freshly prepared serum is used in the tests. The agglutinin for the strain used in immunization generally persists longer than that for other scarlatinal strains. On the other hand, the homologous reaction sometimes tends to diminish at a time when the serum still agglutinates heterologous scarlatinal strains strongly. In all probability this is due to the gradual loss of the agglutinable properties of the strain itself. Very few reactions in which there is any question of specificity occur when sera are prepared and used in the manner described. The results of the study of the agglutination reactions of the strains described are presented in Table I.

Table I is an arrangement of the agglutination reactions with strains of hemolytic streptococci obtained from cases of scarlet fever occurring in New York (N Y 1, 2, 3, 4, and 5), San Francisco (S F 1 and 2), Chicago (Chi 1 and 2), Baltimore (Balt 1), and Copenhagen, Denmark (Den 1 and 2), during the winter of 1922–23. Through the courtesy of the New York State Department of Health we are able to include reactions with strains isolated from the throats of patients and from milk (E 2340 to E 2406, and E 2950) during a milk-borne epidemic of scarlet fever. Strains V 1 and V 2 isolated from throat cultures of patients during the Boston epidemic of septic sore throat were obtained from The Rockefeller Institute for Medical Research. All these strains except Strain E 2950, the milk strain, were obtained from throat cultures. Strains of pyogenic cocci, A, B, C, D, E, F, and G, are non-scarlet strains isolated from infected wounds.

These strains are arranged with the corresponding immune sera in groups according to the sources from which they were obtained.

Each reaction, represented by one of the numerals 1, 2, 3, or the letter C., is the result obtained by agglutinating with two immune sera.

TABLE I.

Cross-Agglutination of Strains of Streptococci Isolated from Clinical Cases of Scarlet Fever.

Strains.	Sera.														
	N Y 5.	N Y 4.	N Y 3.	N Y 2.	N Y 1.	Balt 1.	Den 2.	Den 1.	S F 2.	S F 1.	Chi 2.	Chi 1.	E 2950.	E 2344	E 2340
E 2340	C.	C.	3	3	2	2	—	3	C.	C.	2	C.	C.	C.	C.
E 2341	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—
E 2342	—	—	—	—	—	2	—	—	—	C.	—	—	—	—	—
E 2344	C.	C.	3	2	2	2	—	C.	C.	3	3	C.	2	C.	C.
E 2346	—	—	—	—	—	1	—	—	2	C.	—	—	—	—	—
E 2402	—	—	—	—	—	2	—	—	C.	3	—	3	—	—	—
E 2404	—	—	—	—	—	C.	—	—	—	2	—	—	—	—	—
E 2406	—	—	—	—	—	—	—	—	—	C.	—	—	—	—	—
E 2950	C.	C.	3	2	2	2	—	C.	0	1	3	2	C.	C.	C.
Chi 1	—	—	—	—	—	—	C.	—	C.	C.	3	2	3	—	—
Chi 2	C.	C.	C.	3	C.	C.	"	3	"	3	3	3	C.	C.	C.
S F 1	—	—	—	—	—	—	"	—	"	C.	—	—	"	—	—
S F 2	C.	C.	C.	C.	C.	—	"	3	3	"	—	—	"	C.	C.
Den 1	"	"	"	3	3	2	—	C.	3	2	3	C.	1	—	"
Den 2	1	"	3	C.	0	2	C.	2	3	2	0	2	3	—	—
Balt 1	—	—	—	—	—	C.	"	—	2	C.	—	—	3	—	—
N Y 1	0	3	2	0	C.	3	—	—	1	2	0	1	2	0	1
N Y 2	C.	C.	C.	3	3	C.	—	3	3	3	0	3	1	—	—
N Y 3	"	"	1	3	C.	2	—	2	2	2	2	C.	3	1	1
N Y 4	2	"	C.	1	"	0	—	C.	3	2	0	"	1	1	1
N Y 5	3	—	"	3	"	C.	—	3	2	2	1	"	C.	C.	C.
A	—	—	—	—	—	0	—	0	0	0	—	0	—	—	—
B	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0
D	0	0	0	0	1	0	0	—	0	0	—	0	0	0	0
E	0	0	0	0	1	0	0	—	0	0	—	0	0	0	—
F	0	0	0	0	1	—	0	—	—	—	—	—	0	0	0
G	0	0	0	0	2	—	—	—	—	—	—	—	0	—	—
V 1	0	0	1	—	—	—	0	0	0	0	0	0	0	0	0
V 2	0	0	0	0	—	—	1	—	0	0	0	0	0	1	0

Since these immune sera usually agglutinate the antigenic strains as heavily as they agglutinate other strains of the scarlet group, we have attempted to obtain sera of uniform titer by bleeding animals

at a time during immunization when complete agglutination occurs in dilutions of 1:1,280 and a + + + reaction in dilutions of 1:2,560 with the antigenic strains. This agglutination is designated by the letter C. in the table. The numerals 2 and 3 indicate reactions which are complete as high as 1:320 or 1:640 respectively. When the reaction is not complete in any of the dilutions or complete only in the lower tubes the degree of reaction is indicated by the numeral 1. Completely negative reactions are indicated by zeros. When no agglutination reaction was carried out with certain sera and strains in the cross-agglutination table, the fact has been noted by a minus sign. No reactions are included in the table in which the control tubes show more than a + reaction in a 1:80 dilution of normal rabbit serum.

These streptococcal immune sera are found to agglutinate all of the scarlet strains and with the exception of Serum N Y 1, no agglutination occurs with strains of pyogenic cocci. These reactions confirm the previously reported work of Dochez and Bliss (1), Bliss (2), Gordon (3), and Tunnicliff (4), and substantiate their claim that strains of hemolytic streptococci obtained from scarlatina differ biologically from pyogenic streptococci. On account of the wide distribution of the cases from which these strains were obtained, we believe that this group of streptococci is generally associated with scarlet fever, and that the streptococci with which these authors worked are identical.

Considerable importance is attached to the agglutination of Strains E 2340 to E 2950 since they were obtained both from patients and from the milk during a milk-borne epidemic of scarlet fever. We find that these strains are agglutinated by sera prepared by immunizing animals with other scarlet strains and that sera prepared with these strains agglutinate other streptococci of the scarlatinal group. These reactions identify them as scarlatinal strains and indicate that milk may be a source of contagion in scarlet fever. Strains V 1 and V 2 do not agglutinate with any of the sera, showing that epidemics of streptococcus angina although they are spread through milk infection are caused by a streptococcus differing biologically from *Streptococcus scarlatinæ*.

These strains obtained from the two epidemics all have a final hydrogen ion concentration of less than pH 5.1, indicating an infection of the milk with human rather than bovine strains. The antigenic properties of these strains vary greatly. Certain strains, such as N Y 4 and 5, when used for immunization, always produce sera which agglutinate scarlatinal strains strongly, while other strains such as Chi 2 have given poor or mediocre sera. Certain strains likewise agglutinate more readily and more heavily than others of the group. For example, Chi 2, which has poor immunizing qualities, is agglutinated more strongly than any other strain. Aside from these permanent variations in the antigenic properties strains at times become inagglutinable. This occurs especially after a number of transfers in broth. A number of the 0 reactions in the table can be explained in this way. These inagglutinable strains are still capable of stimulating the production of specific agglutinins in animals.

We have found that certain combinations of sera and strains are necessary for the successful absorption of agglutinin. These combinations can usually be picked out by the speed of the reaction and the degree of agglutination. Strains will absorb the agglutinin from sera which are fresh if the agglutination is complete within 15 minutes at 55°C.

By selecting sera and strains which have reacted vigorously we have been able to obtain a series of absorption tests in which every strain is represented. The serum is diluted 1:40 and absorbed twice at 55°C. with the centrifuged bacteria from 250 cc. of culture. In Tables II to VII the results have been tabulated with the series of dilutions, showing the results of agglutination of a serum and the antigenic strain with the serum after heating at 55°C. and after absorption with scarlet and non-scarlatinal streptococci.

TABLE II.

Absorption of Agglutinin. Serum E 2950 Absorbed with Strains E 2950, B 1, S F 1, S F 2, Chi 1, and Non-Scarlet Strain A.

Serum.	Strain.	Titer of serum with Strain E 2950. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560
Control, normal serum		0	0	0	0	0	0
Immune Serum E 2950		4	3	3	3	2	1
Heated " E 2950		2	3	2	2	2	2
Serum E 2950 absorbed with	E 2950	0	0	0	0	0	0
" E 2950 " "	B 1	0	±	0	0	0	0
" E 2950 " "	S F 1	2	±	0	0	0	0
" E 2950 " "	S F 2	0	±	0	0	0	0
" E 2950 " "	Chi 1	0	1	0	0	0	0
" E 2950 " "	A	2	3	3	±	0	0

TABLE III.

Absorption of Agglutinin. Serum E 2950 Absorbed with Scarlatina Strains E 2950, E 2340, E 2344, N Y 2, N Y 3, and Non-Scarlet Strain A.

Serum.	Strain.	Titer of serum with Strain E 2950. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560
Control, normal serum		0	±	0	0	0	0
Immune Serum E 2950		3	3	3	3	3	1
Heated " E 2950		2	2	2	2	±	0
Serum E 2950 absorbed with	E 2950	1	±	0	0	0	0
" E 2950 " "	E 2340	2	±	0	0	0	±
" E 2950 " "	E 2344	1	2	±	0	0	0
" E 2950 " "	N Y 2	2	2	1	0	0	0
" E 2950 " "	N Y 3	0	0	0	0	0	0
" E 2950 " "	A	2	3	2	±	0	0

TABLE IV.

Absorption of Agglutinin. Serum S F 1 Absorbed with Scarlet Strains S F 1, S F 2, B 1, and Non-Scarlet Strain C.

Serum.	Strain.	Titer of serum with Strain S F 1. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1,2,560
Normal serum control		0	0	0	0	0	0
Immune " S F 1		4	4	4	4	4	3
Heated " S F 1		3	4	4	4	3	2
Serum S F 1 absorbed with	S F 1	0	0	0	0	0	0
" S F 1 " "	S F 2	0	0	0	0	0	0
" S F 1 " "	B 1	0	0	0	0	0	0
" S F 1 " "	C	3	3	3	2	±	0

TABLE V.

Absorption of Agglutinin. Immune Serum Chi 2 Absorbed with Scarlet Strains Chi 1, Chi 2, N Y 1, and Non-Scarlet Strain C.

Serum.	Strain.	Titer of serum with Strain Chi 2. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560
Normal serum control		0	0	0	0	0	0
Heated " Chi 2		±	3	4	3	2	1
Serum Chi 2 absorbed with	Chi 1	1	±	0	0	0	0
" Chi 2 " "	Chi 2	±	0	0	0	0	0
" Chi 2 " "	N Y 1	2	3	3	2	2	0
" Chi 2 " "	C	2	3	3	3	1	0

TABLE VI.

Absorption of Agglutinin. Immune Serum B 1 Absorbed with Scarlet Strains Balt 1, S F 1, S F 2, Den 2, and Non-Scarlet Strain G.

Serum,	Strain.	Titer of serum with Strain Balt 1. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560
Normal serum control		0	0	0	0	±	0
Serum Balt 1		4	4	3	3	2	0
Heated Serum Balt 1		3	3	2	2	1	1
Serum Balt 1 absorbed with	Balt 1	1	0	0	0	0	0
" Balt 1 " "	S F 1	±	0	0	0	0	0
" Balt 1 " "	S F 2	2	1	1	0	0	0
" Balt 1 " "	Den 2	1	0	0	0	0	0
" Balt 1 " "	G	2	3	2	1	0	0

TABLE VII.

Absorption of Agglutinin. Serum Den 2 Absorbed with Scarlet Strains Den 1, Den 2, and Non-Scarlet Strain D.

Serum.	Strain.	Titer of serum with Strain Den 2. Dilutions.					
		1:80	1:160	1:320	1:640	1:1,280	1:2,560
Normal serum control		0	0	0	0	0	0
Serum Den 2		3	4	4	3	2	±
Heated Serum Den 2		2	2	3	2	0	0
Serum Den 2 absorbed with	Den 2	0	0	0	0	0	0
" Den 2 " "	Den 1	±	0	0	0	0	0
" Den 2 " "	D	+	3	2	2	±	0

CONCLUSIONS.

1. Strains of hemolytic streptococci from cases of scarlet fever occurring in New York, San Francisco, Chicago, Baltimore, and Copenhagen, Denmark, all interagglutinate with immune sera prepared with these strains.

2. Sera prepared with these strains do not agglutinate pyogenic streptococci or strains isolated from cases of septic sore throat.

3. The strains obtained from the throats of patients from an epidemic of scarlet fever and the strain from the milk responsible for this epidemic fall into the scarlatinal group according to these agglutination tests.

4. Absorption tests can be carried out with these strains and sera under proper conditions.

5. A group of hemolytic streptococci biologically distinct from streptococci from other sources than scarlet fever is constantly associated with scarlatina. They constitute a group of closely related streptococci which may be identified by agglutination tests.

We are indebted to Dr. Matthias Nicoll and Dr. A. B. Wadsworth of the New York State Department of Health, to Dr. Ruth Tunnicliff of Chicago, to Miss Eugenia Valentine of San Francisco, and to Dr. Vilk. Jensen of Copenhagen for cultures of *Streptococcus scarlatinæ*.

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THE INHIBITORY EFFECT OF ACRIDINE ON THE SPOROGONY OF A COCCIDIUM (EIMERIA STIEDÆ).

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(Received for publication, April 4, 1924.)

The common coccidium of the rabbit is transmitted from animal to animal through the medium of an encysted stage which is deposited with the feces and then undergoes a period of ripening during which the enclosing membrane remains intact. The cyst is then capable of infesting fresh animals and probably of reinfesting its own host. The ripening process consists in the development within the oocyst of four sporoblasts, then of four spores from these, and finally of eight sporozoites by the internal division of each spore into two.

The oocyst is characterized by a heavy, double contoured wall which appears to be a very effective protection for the protoplasmic structures within. The development proceeds in the presence of a reasonable amount of moisture and at ordinary summer temperatures under a wide range of surrounding conditions. Heavy cultures of the yeasts, molds, and bacteria of rabbit feces may develop about the cysts without interfering in the slightest with their ripening. Free oxygen is said to be essential to the process. It is also said that considerable concentrations of the common disinfectants are withstood.

The coccidium disease of the rabbit is a serious one for those who attempt to raise this animal, as under otherwise good conditions it often causes a very high mortality among the young. The disease is also the prototype of diseases in cattle and poultry which frequently occasion considerable losses. Human beings are sometimes affected. In the rabbit the disease consists of an infestation of the epithelial cells of the small intestine and the bile ducts in the liver. In other

animals the liver is not invaded and in the rabbit it is by no means always involved. The liver has been involved in some human cases. Spread of the disease occurs within the intestinal tract through the medium of merozoites which are for a longer or shorter time free in the contents. It would seem not unlikely that the parasites, in this stage at least, might be more than ordinarily accessible to the action of drugs. However, when one considers what is known of coccidial disease from the point of view of treatment, the available information is meager in the extreme. In the fatal cases the oocysts of the parasite are present in the feces in numbers, at times it is stated in enormous numbers. The more severe stages of the disease are apt to be marked by a diarrhea and in cattle this is frequently bloody.

The length of time occupied by the earlier stages of the disease is not known. Nor is it known whether the number of oocysts in the feces varies in such a way as to give any clue to the progress of the disease in the host. There is no other test which may be applied to control progress.

In the light of this general situation it was thought that further study might be of eventual help and, as one step, that it would be of interest to see whether certain chemical compounds which are known to act against other protozoa, either in the test-tube or therapeutically, would have an influence on the ripening process of the oocysts of the rabbit's coccidium. Observations on this point seem not to have been reported previously.

Methods.—Feces or the contents of the large intestine were rubbed in water to make a heavy suspension. This was, when necessary, strained through a layer of fine gauze to remove the larger fragments of vegetable fiber. The cloudy suspension was mixed with an equal volume of a saturated solution of cane-sugar, shaken well, and centrifuged. (International Centrifuge, Size 2, Type B, rheostat set at 16, time 15 minutes.) The oocysts are thus concentrated at the top of the tube and were removed by first stirring the top centimeter of fluid and then dipping into it a large wire loop of the type used for transferring bacterial cultures. Owing to peculiar adhesive qualities of the cysts they tend to come away with the first few loopfuls. It was found advantageous to prepare several small tubes rather than one large one. With material containing cysts in abundance it is possible to dip ten or fifteen times in each tube and get many cysts each time. After this they become noticeably less abundant.

Agar was dissolved in water to make a 2 per cent gel. This was tubed, 9 cc. per tube, and sterilized. For use this was poured into Petri dishes, a loopful or more of the suspension was placed on the surface at a marked spot after solidification. This preparation was kept in a warm room (between 70° and 80°F.), in the dark, during the observation period. The plates were opened daily and a drop or two of water put on the planted spot. The method of concentration described is adapted from that proposed by Sheather[1] for eggs of parasites in feces. The method of culture is essentially that used previously in this laboratory by Smith and Graybill.[2]

The tests of action of the various substances were made by adding them, before pouring the plates, to the melted agar in appropriate quantities together with sufficient water to make 10 cc. in the tube.

Observations of the progress of the cultures was made with the low powers of the microscope through a drop of water. This was checked at critical points by dropping a cover-slip on the culture and observing with higher powers. Cultures under the cover-slip seemed to proceed with much less regularity, probably due to unevenness in the oxygen supply.

The following substances were tested by this method. The figure in parenthesis following the name is the denominator of the dilution fraction of the greatest concentration employed. Thus quinine hydrochloride (100) means that the 1/100 dilution of this substance was observed as a maximum concentration.

1. Compounds known to be active against other protozoa: Quinine HCl (100); tartar emetic (100); *p*-aminophenylarsenoxide (100); emetine (1,000); acridine HCl (10,000); proflavine (1,000); methylene blue (10,000); trypan red (2,000); trypan blue (10,000).

2. Compounds related to acridine:[3] Acridine orange (1,000); acridine red (1,000), phosphine N. Bad. (1,000); chrysaniline Kahl. (1,000); phosphine 3 R (500); phosphine G. (1,000); acridine yellow (1,000); cyanotrypaflavine (1,000); 9-methylacridinium methochloride (1,000); leuco-cyano trypaflavine (1,000); 3-6-diamino-9-phenylacridine (1,000); 3-amino-6-hydroxyacridine dihydrochloride (1,000); 3-6-diaminoacridine (1,000); 3-aminoacridine (1,000); 2-7-diaminocarbazole (1,000); 2-7-diamino-9-phenylacridine (1,000); diaminonaphthacridine, impure, (1,000); 9-phenylacridine (1,000).

3. Other compounds: Safranine$_w$ (2,000); Nile blue sulfate (10,000); methyl violet 6 B (10,000); malachite green (10,000); neutral red (10,000); eosin (2,000);

[1] Sheather, A. L., *J. Comp. Path. and Therap.*, 1923, xxxvi, 71.

[2] Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1918, xxviii, 89.

[3] I am indebted to Dr. W. A. Jacobs, of The Rockefeller Institute, New York, for the unusual compounds in this group as well as for a helpful interest in the problem.

benzidine (saturated); acetanilide (saturated); indazole (saturated); trimethylamine HCl (100); para-phenylendiamine (100); aniline HCl (100); quinoline yellow (1,000); quinoline hydrochloride platinic salt (1,000); isoquinoline hydrochloride platinic salt (1,000); β-naphthoquinoline HCl (1,000).

Of these compounds only one, acridine hydrochloride, has shown any effect. This substance completely inhibits the ripening of most of the oocysts under certain conditions which may be briefly discussed.

In concentration of 1/10,000 development of nearly all the cysts is entirely inhibited. Concentrations down to and including 1/80,000 act in the same way. In concentrations 1/100,000 to 1/1,000,000 development is still inhibited but in lesser and decreasing degree. The inhibition in this range manifests itself in delay and in slower progress in contrast to the more concentrated range where, with certain exceptions, development is completely prevented. The oocysts on the plates in the concentrated range apparently lie dormant. The nuclear matter undergoes no visible change either of development or degeneration.

There is, however, an exception to and also an interesting limitation on these statements. As an exception it is to be noted that an occasional cyst undergoes its normal development regardless of the concentration of acridine to which it is exposed. The sporoblasts are formed with the controls and the ripening process is completed in the usual way and in the usual time. Furthermore, if the oocysts are allowed to stand in a warm room for 24 hours until sporoblasts have been formed and then are planted in the acridine plates the development proceeds normally to the formation of sporozoites.

The exceptional development of an occasional cyst may be an instance of "fastness" such as has been commonly noticed in chemotherapeutic experiments with trypanosomes.

The fact that after sporoblasts are formed acridine no longer has an influence is interesting indicating as it does an action of the compound very precisely directed at a particular condition of the parasite, a particular stage in the parasite's life cycle.

Ehrlich when developing the results of his chemotherapeutic experiments held as closely as possible to the idea that his compounds acted, either as such or in modified form, directly as germicidal

agents against the parasite. Others since have shown that in some instances the action of the compound must be on the host rather than on the parasite; chemicals may stimulate the body cells of the host to the production of antiparasitic substances, or perhaps to other antiparasitic action. This general question of mechanism has been well reviewed recently by Dale.[4]

This action of acridine on the oocysts of the coccidium in the resting stage, contrasted with its failure to act after sporoblasts are formed, presents a nice example of action specifically directed and directly exerted.

SUMMARY.

The development or ripening of the oocyst of the coccidium of the rabbit is prevented by acridine hydrochloride provided that the cysts are exposed to the action of the chemical before development has started. After sporoblasts are formed acridine does not prevent further development. Many other substances, some of them known to be active against certain protozoan parasites, have no influence on the ripening of the oocysts of the coccidium.

[4] Dale, H. H., *Physiol. Rev.*, 1923, iii, 359.

METHEMOGLOBIN FORMATION BY STERILE CULTURE FILTRATES OF PNEUMOCOCCUS.

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(Received for publication, April 4, 1924.)

A constant characteristic of growth of Pneumococcus in media containing blood is the production of a color change in the blood pigment. Gilbert and Fournier (1) (1896) observed that the growth of Pneumococcus in defibrinated rabbit blood causes a greenish brown discoloration of the medium. By spectroscopic examination they ascertained that this was due to the presence of methemoglobin. Much has been written concerning the mechanism of this phenomenon.

Grüter (2) (1909) confirmed the original observation of Gilbert and Fournier and found that the substance responsible for the reaction is extractable from the bacterial bodies; that it is thermolabile; and that it can be demonstrated occasionally, though not constantly, in filtrates of cultures and of bacterial extracts. Butterfield and Peabody (3) (1913) made the first quantitative study of the action of pneumococci on blood, employing spectrophotometric methods. They showed that when blood is acted upon by pneumococci its oxygen capacity is markedly reduced, and concluded that this is due to the transformation of oxyhemoglobin to methemoglobin by the organisms. Although the work of Butterfield and Peabody concerned itself more with the action of the living organism on blood than with the identification or nature of the reactive substance, they found that an active principle, which induces these blood changes, is present in sterile filtrates of autolyzed cultures. On the other hand, Cole (4) (1914) found that no production of methemoglobin occurred when sterile filtrates of pneumococcus cultures were added to hemoglobin solutions prepared by laking red blood cells, and concluded that the phenomenon occurs only in the presence of the living organism. Stadie (5) (1921) also found that methemoglobin was not produced when sterile filtrates of pneumococcus cultures were added to oxyhemoglobin solutions. Rieke (6) (1904), studying a similar type of blood change induced by streptococci, felt that methemoglobin formation is the result of physiological cell function and is intimately associated with the life processes of the organisms themselves, since neither culture

filtrates nor killed organisms produced the reaction. Schnabel (7) (1921) was able to demonstrate the production of methemoglobin by sterile filtrates of blood broth cultures of Pneumococcus.

Recently it has been shown that hydrogen peroxide occurs in cultures of Pneumococcus and that this substance is capable of causing the pigment change (8–10). The compound makes its appearance, under certain well defined conditions, during the logarithmic phase of growth of the cultures and, even in the absence of organisms, is capable of converting solutions of crystalline oxyhemoglobin into methemoglobin (11). This conversion occurs, however, only when the oxyhemoglobin solution contains no catalase.

Despite the disagreement among earlier workers concerning methemoglobin formation in the absence of the living cell, Neill and Avery (12) (1924) have conclusively shown that this reaction may be brought about by sterile extracts of Pneumococcus. Extracts were prepared from anaerobically grown pneumococci by repeatedly freezing and thawing the cells. Throughout the preparation the extracts were protected from air, and were finally filtered in a nitrogen atmosphere. These sterile preparations, if stored in the absence of oxygen, possessed the power of forming peroxide when exposed to air (13), of actively reducing methylene blue (14), of consuming molecular oxygen (12), of oxidizing pneumococcus hemotoxin (15), and of converting to methemoglobin the oxyhemoglobin prepared from laked red blood cells (12). Methemoglobin formation by these extracts seemed dependent upon the interaction of two distinct factors, each of which is separably inactive. One of these, an intracellular, labile substance, is present in the saline extracts of washed cells. The other factor essential to the system is heat-stable and may be obtained from non-bacterial sources, as yeast extract and meat infusion. From the experimental evidence available, it is inferred that methemoglobin formation by this system involves an initial deoxygenation of oxyhemoglobin and a union of free oxygen with some autoxidizable substance. The peroxide thus formed serves as the actual oxidizing agent in the conversion of the hemoglobin to methemoglobin. The mechanism of methemoglobin formation by pneumococcus extracts is interpreted as an oxidation process in which deoxygenation of oxyhemoglobin and peroxide formation occur as intermediary reactions. The active agent of the oxidation of hemoglobin is considered to be a peroxide of bacterial origin. The systems responsible for methemoglobin formation and the other oxidation-reduction processes were found to retain their activity for long periods of time if the extracts were protected from air. Upon exposure to atmospheric oxygen, however, their activity is rapidly destroyed. This loss of activity is apparently due to the action of peroxide or other oxidizing agents which are formed in the extract when oxygen is available.

From the facts presented in previous reports (10, 12–16) it becomes evident that the following considerations must be kept in mind in an investigation of the phenomenon of methemoglobin for-

mation by the filtrates of pneumococcus cultures. (1) Hydrogen peroxide accumulates during the active, aerobic growth of Pneumococcus and also during the oxidation of sterile extracts of Pneumococcus. Methemoglobin formation by concentrations of hydrogen peroxide such as are present under these circumstances occurs only in the absence of blood catalase. (2) A labile constituent of the bacterial cell is concerned in the conversion of catalase-containing solutions of oxyhemoglobin by sterile pneumococcus extracts. This cell constituent is itself susceptible to oxidizing agents and may be rendered inactive if exposed to peroxides or similar substances, previous to its introduction into oxyhemoglobin solutions.

With these distinctions in mind, the conditions essential for methemoglobin formation by sterile culture *filtrates* may be predicted with some degree of certainty. If a culture of Pneumococcus is filtered during the logarithmic phase of growth, the filtrate will contain only soluble products of growth and little or none of the actual intracellular substances. One soluble product of growth is hydrogen peroxide and it is known that this substance causes the formation of methemoglobin from crystalline oxyhemoglobin in the absence of catalase. The occurrence of peroxide is dependent upon the free access of air to the culture fluid. If cultures are grown under anaerobic conditions, this substance is not formed. On the other hand, filtrates of cultures which have passed the period of active growth and are undergoing autolysis will contain those intracellular substances which are liberated during cell disintegration. It has been shown that hydrogen peroxide destroys certain of the intracellular substances in pneumococcus extracts. It would seem, then, that in attempting to demonstrate the activity of the intracellular constituents free in the fluid of autolyzing pneumococcus cultures, care should be taken to prevent the formation of peroxide during growth. The ability of culture filtrates to form methemoglobin in the presence of catalase may depend, therefore, not only upon whether the filtrates contain the necessary intracellular substances, but also upon whether these substances have been protected from products of oxidation (peroxides) which are known to destroy them. In order to determine the validity of this hypothesis

a comparative study has been made of filtrates of young unautolyzed and of old autolyzed cultures, grown both anaerobically and aerobically.

Materials.

Hemoglobin Solutions.—Two preparations of hemoglobin were used. The first was made by laking red blood corpuscles in distilled water; the hemoglobin solution was diluted in phosphate solution pH 7.5 to a final hemoglobin concentration of 1 mM. The second preparation consisted of a solution of crystalline hemoglobin, catalase-free, in phosphate solution pH 7.8. The final hemoglobin concentration was 1 mM.[1]

Media and Cultures.—Plain broth pH 7.8 was employed. For anaerobic cultures the incubation was carried out in an anaerobic jar or the medium sealed with a thick layer of melted vaseline. Stock strains of pneumococci of Types I, II, and III and of Group IV were used.

EXPERIMENTAL.

Methemoglobin Formation by Filtrates of Actively Growing Cultures.—In agreement with the observations of Cole it was found that bacteria-free filtrates of young unautolyzed cultures of Pneumococcus, grown aerobically or anaerobically, do not possess the ability to form methemoglobin from solutions of oxyhemoglobin prepared from laked red blood cells containing catalase.

It has been shown that, under optimal conditions, hydrogen peroxide is demonstrable during the logarithmic period of growth and that simultaneously with its occurrence the bacteria-free filtrate of the culture acquires the property of changing crystalline hemoglobin, containing no catalase, to methemoglobin (11). A further study was made of the relation between the presence of peroxide and methemoglobin formation by filtrates of young cultures. To prevent the occurrence of demonstralbe peroxide in the culture fluids the organisms were grown both anaerobically and in the presence of plant tissue. As a control, to demonstrate the action of peroxide-containing filtrates, a third culture was grown in broth with free access

[1] Unless otherwise stated, the tests for methemoglobin formation were made by adding 0.5 cc. of the filtrates to be tested to 0.5 cc. of the hemoglobin solution and incubating the mixture for 2 hours at 37°C. in the water bath.

of air; that is, under circumstances favoring peroxide production. The three cultures were passed separately through Berkefeld filters and the filtrates immediately added to solutions of catalase-free hemoglobin.

Experiment 1.—50 cc. of plain broth pH 7.8 were placed in each of three 250 cc. Erlenmeyer flasks. To one of these flasks pieces of sterile unheated potato were added (17, 18). The flasks were then seeded with 0.5 cc. of an 8 hour plain broth culture of Pneumococcus Type II. The culture containing plant tissue (Culture B) and one of the plain broth cultures (Culture A) were incubated at 37°C. at atmospheric oxygen tension. The other culture in plain broth (Culture C) was incubated under anaerobic conditions in Brown's modified anaerobic jar. At the end of 8 hours incubation, the cultures were filtered and tests were performed for the presence of peroxide[2] and for methemoglobin-forming activity. The results of the experiment are given in Table I.

TABLE I.

Relation of the Presence of Peroxide in Pneumococcus Filtrates to the Formation of Methemoglobin from Catalase-Free Oxyhemoglabin (Crystalline).

Broth culture of Pneumococcus Type II.	Age of culture.	Filtrate.	
		Peroxide test.	Methemoglobin formed from catalase-free oxyhemoglobin (crystalline).
	hrs. 37°C.		
Aerobic (A).	8	Positive.	+
" (B) (with vegetable tissue).	8	Negative.	−
Anaerobic (C).	8	"	−

From the results given in Table I, it is clear that Berkefeld filtrates of cultures of Pneumococcus actively growing under conditions unfavorable for the formation or accumulation of peroxide, do not possess the ability of transforming catalase-free oxyhemoglobin into methemoglobin.

The thermolability of peroxide in broth culture filtrates has been discussed in a previous paper (10). It has been shown that when a

[2] The test for peroxide used in this work is as follows: 0.5 cc. of the fluid to be tested is placed in a small test-tube. A piece of fresh unheated potato, 1 by 0.5 cc., is utilized as the peroxidase. This is immersed in the fluid and 2 or 3 drops of freshly prepared saturated glacial acetic acid solution of benzidine are added. The development of a blue color indicates the presence of peroxide.

peroxide-containing filtrate is subjected to 100°C., the peroxide rapidly disappears and that after the destruction of the peroxide the filtrate no longer possesses the property of forming methemoglobin.

Since the filtrates of young aerobic cultures behave, as regards the benzidine test, like dilute solutions of hydrogen peroxide, the methemoglobin-forming property of this reagent was tested, various concentrations in broth being used. It was found that hydrogen peroxide, when added to broth, causes methemoglobin formation in solutions of oxyhemoglobin containing no catalase. This phenomenon parallels the presence of the benzidine test for peroxide; both reactions occur when broth contains 0.003 per cent H_2O_2. Faint reactions were obtained with broth containing 0.002 per cent H_2O_2.

To summarize, it has been shown that filtrates of young, unautolyzed pneumococcus cultures, grown either aerobically or anaerobically, do not possess the ability to convert a solution of oxyhemoglobin, prepared from laked red blood cells and containing, therefore, abundant catalase, into methemoglobin. However, aerobic cultures grown under conditions favorable for the formation and accumulation of hydrogen peroxide yield filtrates which convert a solution of catalase-free oxyhemoglobin to methemoglobin. This pigment change is dependent upon the peroxide in such filtrates.

Methemoglobin Formation by Filtrates of Autolyzing Cultures.—When pneumococcus cultures are grown with a large surface area of culture fluid directly exposed to air, and allowed to autolyze under aerobic conditions, no substance accumulates in the culture fluid which is capable of converting an oxyhemoglobin solution containing catalase into methemoglobin.

When, by the use of anaerobic methods, the accumulation of oxidation products (peroxides) is prevented, a substance, or substances, appear free in the autolyzing fluid which, even in the presence of the catalase of red blood corpuscles, cause methemoglobin formation. The following experiment was devised to show whether the substance, or substances, which are responsible for the formation of methemoglobin in the presence of abundant catalase are liberated only upon autolysis of the cells and whether they can be demonstrated only when precautions are taken to prevent their destruction by oxidation products which accumulate in aerobic cultures.

100 cc. of plain broth were placed in each of three 500 cc. Erlenmeyer flasks and in each of three 100 cc. Florence flasks. In the Erlenmeyer flasks the broth reached a depth of about 3 cm., while in the Florence flasks the medium completely filled the vessels to the neck. The latter were placed in boiling water for 15 minutes, sealed with 5 cc. of melted vaseline, and immediately cooled. Each flask was seeded with 0.5 cc. of a 6 hour plain broth culture of Pneumococcus Type II. Thus, three aerobic and three anaerobic cultures were obtained.

After 8 hours incubation, at a time when growth was at its height, one aerobic and one anaerobic culture were removed from the incubator and passed through a Berkefeld filter. In the filtration of the anaerobic culture care was taken to avoid excessive aeration by carrying out the procedure in an atmosphere of nitro-

TABLE II.

Methemoglobin Formation in the Presence of Blood Catalase by Pneumococcus Filtrates.

Broth culture of Pneumococcus Type II.	Age of culture.	Filtrate.	
		Peroxide test.	Methemoglobin formed in solutions of laked blood cells (containing catalase).
	hrs. 37°C.		
Aerobic.	8	Positive.	−
	72	"	−
	192	Negative.*	−
Anaerobic.	8	"	−
	72	"	+
	192	"	+

*The peroxide formed in aerobic cultures is unstable and may be no longer demonstrable after 6 to 12 days of incubation.

gen. 0.5 cc. quantities of each of the filtrates were placed in tubes containing an equal volume of a hemoglobin solution freshly prepared from laked rabbit blood cells. The tests were incubated for 1 hour in a water bath at 37° C. The presence or absence of methemoglobin was then determined by macroscopic and spectroscopic examinations.

Similarly, at the end of 72 and 192 hours incubation the remaining aerobic and anaerobic cultures were examined in the same manner.

The results of the experiments are given in Table II.

From Table II it is seen that as in preceding experiments, no methemoglobin formation results when filtrates of either young or autolyzing aerobic cultures are added to hemoglobin solutions con-

taining catalase. Likewise, the filtrates of young anaerobic cultures of Pneumococcus do not contain methemoglobin-forming substances. However, when a pneumococcus culture is allowed to proceed to the stage of autolysis under anaerobic conditions a substance, or substances, appear in the filtrate which convert oxyhemoglobin into methemoglobin independent of the presence of catalase.

It has been previously noted (16) that variations occur in the readiness with which different strains of pneumococci form peroxide and that these differences are reflected in the methemoglobin-forming properties of sterile extracts prepared from these strains (12). Similar quantitative variations have been noted in the study of methemoglobin formation by sterile autolysates. It was found that with certain strains of pneumococci, 0.1 cc. of the filtered autolysate of an anaerobic culture sufficed to convert 0.5 cc. of the standard hemoglobin solution to methemoglobin, while with other strains the addition of five times this amount was necessary before the phenomenon became demonstrable. It is noteworthy that the autolysates most active in this respect were derived from strains of organisms which, during growth, formed peroxide more abundantly, and which, when their cells were put into solution by artificial methods, as freezing and thawing, gave the most active methemoglobin-forming extract.

DISCUSSION.

A review of the literature on the subject of methemoglobin formation by pneumococci reveals a lack of agreement concerning both the interpretation of this phenomenon and the definition of the conditions under which it occurs. Attempts to demonstrate this pigment change with sterile culture filtrates have led, until recently, to irregular or negative results. A study of the phenomenon has been presented in recent articles from this laboratory. One of these deals with methemoglobin formation by sterile extracts of Pneumococcus (12); the other with methemoglobin formation by sterile culture filtrates (11). In the light of this work it seemed advisable to restudy the phenomenon directing attention especially to (1) cultural conditions such as the age of the culture and the oxygen tension of the medium and (2) the influence of the presence or absence of catalase in the oxyhemoglobin.

The transformation of oxyhemoglobin to methemoglobin has been demonstrated with the cell-free filtrates of cultures grown under two distinct sets of conditions, (1) young, well aerated cultures and (2) autolyzed, anaerobic cultures.

Under the first set of cultural conditions, the organisms are in the active phase of cell growth, undergoing little or no autolysis; the medium is fully exposed to air during growth; *i.e.*, the ratio of surface area to total volume of culture fluid is large. This results in the formation of hydrogen peroxide which has been shown to change catalase-free oxyhemoglobin to methemoglobin. However, when a solution of laked blood cells is employed, the peroxide in the filtrate is promptly destroyed by the blood catalase and methemoglobin is not formed. It seems unlikely, therefore, that this type of reaction, dependent solely upon the presence of preformed peroxide in this filtrate, and operative only in the absence of blood catalase, represents the actual mechanism by which the transformation of oxyhemoglobin to methemoglobin is effected by the bacterial cell itself.

It has been shown that under the second set of cultural conditions, there is present in the filtrates a system which is indifferent to blood catalase and which converts oxyhemoglobin to methemoglobin, even when the former is prepared from laked red blood corpuscles. This system includes a substance which is endocellular in origin and labile in nature, and is demonstrable only in filtrates of cultures which are autolyzing and which have been maintained under greatly reduced oxygen tension or under strictly anaerobic conditions. As might be expected, these filtrates behave in a manner identical with that of the sterile extracts prepared by Avery and Neill. The intracellular substances were obtained in solution in the extracts by artificially rupturing the walls of young cells; they are present in the filtrates of cultures as a result of cell autolysis. When the pneumococcus cells are removed (by filtration) from the culture fluid before autolysis has occurred, the filtrate does not contain these endocellular substances. It seems likely then that *extracts* of pneumococcus cells and *filtrates* of autolyzed pneumococcus cultures differ chiefly in the concentration of the endocellular substances. Methemoglobin is formed by both preparations only when the hemoglobin is present

during the oxidations induced by these substances, for if, as in the case of the bacterial extracts, the endocellular substances undergo oxidation before the addition of blood, no pigment change results.

The complete methemoglobin-forming system of Pneumococcus extracts has been shown to consist of two factors. One is thermolabile and derived from the pneumococcus cell; the other is thermostable and not necessarily of pneumococcus origin. Since the latter is contained in meat infusion, it was furnished by the medium employed in the present work. As in the case of other endocellular substances of Pneumococcus (*viz.*, the enzymes and hemotoxin) the thermolabile component of the methemoglobin-forming system is liberated in the culture fluid during cell autolysis. Although this system is liberated during autolysis of both aerobic and anaerobic cultures, in the former instance it is rapidly destroyed by the oxidizing agents (peroxides) which are formed as a result of oxygen exposure. Under anaerobic conditions, on the other hand, the formation of oxidizing agents does not occur; the thermolabile component is not destroyed, and, when complemented by the stable factor, these substances together constitute an oxidation-reduction system which endows the filtrate with the methemoglobin-forming function. It seems likely that this system is responsible for methemoglobin formation not only by extracts and autolysates of pneumococcus cells, but by the living organism itself.

CONCLUSIONS.

1. Sterile filtrates of aerobic cultures of pneumococcus which contain hydrogen peroxide are capable of converting catalase-free solutions of crystalline oxyhemoglobin into methemoglobin. This action is dependent upon the hydrogen peroxide in the filtrates and occurs only in the absence of blood catalase.

2. In catalase-containing solutions of hemoglobin from laked corpuscles, the actual methemoglobin-forming system of Pneumococcus involves a labile constituent of the bacterial cell. This intracellular substance is itself susceptible to oxidizing agents and may be rendered inactive, if exposed to peroxide or similar substances previous to its introduction into oxyhemoglobin solutions. The activity of this function in the case of sterile filtrates of pneumococcus cultures

depends, therefore, upon the liberation of cell constituents into the medium and upon the protection of these cellular substances from the oxidizing agents which are formed when pneumococcus cultures are freely exposed to air. When these cultural conditions are fulfilled, sterile culture filtrates of Pneumococcus convert oxyhemoglobin into methemoglobin independent of the presence of blood catalase.

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